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(54) Title: COMPOSITIONS AND METHODS FOR THE TREATMENT AND DIAGNOSIS OF CARDIOVASCULAR DISEASE		
(57) Abstract <p>The present invention relates to methods and compositions for the treatment and diagnosis of cardiovascular disease, including, but not limited to, atherosclerosis, ischemia/reperfusion, hypertension, restenosis, and arterial inflammation. Specifically, the present invention identifies and describes genes which are differentially expressed in cardiovascular disease states, relative to their expression in normal, or non-cardiovascular disease states, and/or in response to manipulations relevant to cardiovascular disease. Further, the present invention identifies and describes genes via the ability of their gene products to interact with gene products involved in cardiovascular disease. Still further, the present invention provides methods for the identification and therapeutic use of compounds as treatments of cardiovascular disease. Moreover, the present invention provides methods for the diagnostic monitoring of patients undergoing clinical evaluation for the treatment of cardiovascular disease, and for monitoring the efficacy of compounds in clinical trials. Additionally, the present invention describes methods for the diagnostic evaluation and prognosis of various cardiovascular diseases, and for the identification of subjects exhibiting a predisposition to such conditions.</p>		

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COMPOSITIONS AND METHODS FOR THE TREATMENT
AND DIAGNOSIS OF CARDIOVASCULAR DISEASE

This application claims the benefit under 35 U.S.C. §119(e) of co-pending provisional Application No. 60/011,787 filed February 16, 1996.

1. INTRODUCTION

The present invention relates to methods and compositions for the treatment and diagnosis of cardiovascular disease, including, but not limited to, atherosclerosis, ischemia/reperfusion, hypertension, restenosis, and arterial inflammation. Genes which are differentially expressed in cardiovascular disease states, relative to their expression in normal, or non-cardiovascular disease states are identified. Genes are also identified via the ability of their gene products to interact with other gene products involved in cardiovascular disease. The genes identified may be used diagnostically or as targets for therapeutic intervention. In this regard, the present invention provides methods for the identification and therapeutic use of compounds in the treatment and diagnosis of cardiovascular disease. Additionally, methods are provided for the diagnostic monitoring of patients undergoing clinical evaluation for the treatment of cardiovascular disease, for monitoring the efficacy of compounds in clinical trials, and for identifying subjects who may be predisposed to cardiovascular disease.

2. BACKGROUND OF THE INVENTION

Cardiovascular disease is a major health risk throughout the industrialized world. Atherosclerosis, the most prevalent of cardiovascular diseases, is the principal cause of heart attack, stroke, and gangrene of the extremities, and thereby the principal cause of death in the United States. Atherosclerosis is a complex disease involving many cell types and molecular factors (for a detailed review, see Ross, 1993, Nature 362: 801-809). The

process, in normal circumstances a protective response to insults to the endothelium and smooth muscle cells (SMCs) of the wall of the artery, consists of the formation of fibrofatty and fibrous lesions or plaques, preceded and
5 accompanied by inflammation. The advanced lesions of atherosclerosis may occlude the artery concerned, and result from an excessive inflammatory-fibroproliferative response to numerous different forms of insult. For example, shear stresses are thought to be responsible for the frequent
10 occurrence of atherosclerotic plaques in regions of the circulatory system where turbulent blood flow occurs, such as branch points and irregular structures.

The first observable event in the formation of an atherosclerotic plaque occurs when blood-borne monocytes
15 adhere to the vascular endothelial layer and transmigrate through to the sub-endothelial space. Adjacent endothelial cells at the same time produce oxidized low density lipoprotein (LDL). These oxidized LDL's are then taken up in large amounts by the monocytes through scavenger receptors
20 expressed on their surfaces. In contrast to the regulated pathway by which native LDL (nLDL) is taken up by nLDL specific receptors, the scavenger pathway of uptake is not regulated by the monocytes.

These lipid-filled monocytes are called foam cells,
25 and are the major constituent of the fatty streak.

Interactions between foam cells and the endothelial and SMCs which surround them lead to a state of chronic local inflammation which can eventually lead to smooth muscle cell proliferation and migration, and the formation of a fibrous
30 plaque. Such plaques occlude the blood vessel concerned and thus restrict the flow of blood, resulting in ischemia.

Ischemia is a condition characterized by a lack of oxygen supply in tissues of organs due to inadequate perfusion. Such inadequate perfusion can have number of
35 natural causes, including atherosclerotic or restenotic lesions, anemia, or stroke, to name a few. Many medical interventions, such as the interruption of the flow of blood

during bypass surgery, for example, also lead to ischemia. In addition to sometimes being caused by diseased cardiovascular tissue, ischemia may sometimes affect cardiovascular tissue, such as in ischemic heart disease.

5 Ischemia may occur in any organ, however, that is suffering a lack of oxygen supply.

The most common cause of ischemia in the heart is atherosclerotic disease of epicardial coronary arteries. By reducing the lumen of these vessels, atherosclerosis causes
10 an absolute decrease in myocardial perfusion in the basal state or limits appropriate increases in perfusion when the demand for flow is augmented. Coronary blood flow can also be limited by arterial thrombi, spasm, and, rarely, coronary emboli, as well as by ostial narrowing due to luetic
15 aortitis. Congenital abnormalities, such as anomalous origin of the left anterior descending coronary artery from the pulmonary artery, may cause myocardial ischemia and infarction in infancy, but this cause is very rare in adults. Myocardial ischemia can also occur if myocardial oxygen
20 demands are abnormally increased, as in severe ventricular hypertrophy due to hypertension or aortic stenosis. The latter can be present with angina that is indistinguishable from that caused by coronary atherosclerosis. A reduction in the oxygen-carrying capacity of the blood, as in extremely
25 severe anemia or in the presence of carboxy-hemoglobin, is a rare cause of myocardial ischemia. Not infrequently, two or more causes of ischemia will coexist, such as an increase in oxygen demand due to left ventricular hypertrophy and a reduction in oxygen supply secondary to coronary
30 atherosclerosis.

The principal surgical approaches to the treatment of ischemic atherosclerosis are bypass grafting, endarterectomy, and percutaneous transluminal angioplasty (PCTA). The failure rate after these approaches due to
35 restenosis, in which the occlusions recur and often become even worse, is extraordinarily high (30-50%). It appears

that much of the restenosis is due to further inflammation, smooth muscle accumulation, and thrombosis.

A modified balloon angioplasty approach was used to treat arterial restenosis in pigs by gene therapy (Ohno et al., 1994, Science 265: 781-784). A specialized catheter was used to introduce a recombinant adenovirus carrying the gene encoding thymidine kinase (tk) into the cells at the site of arterial blockage. Subsequently, the pigs were treated with ganciclovir, a nucleoside analog which is converted by tk into a toxic form which kills cells when incorporated into DNA. Treated animals had a 50% to 90% reduction in arterial wall thickening without any observed local or systemic toxicities.

Because of the presumed role of the excessive inflammatory-fibroproliferative response in atherosclerosis and ischemia, a number of researchers have investigated, in the context of arterial injury, the expression of certain factors involved in inflammation, cell recruitment and proliferation. These factors include growth factors, cytokines, and other chemicals, including lipids involved in cell recruitment and migration, cell proliferation and the control of lipid and protein synthesis.

For example, the expression of PDGF (platelet derived growth factor) or its receptor was studied: in rats during repair of arterial injury (Majesky et al., 1990, J. Cell Biol. 111: 2149); in adherent cultures of human monocyte-derived macrophages treated with oxidized LDL (Malden et al., 1991, J. Biol. Chem. 266: 13901); and in bovine aortic endothelial cells subjected to fluid shear stress (Resnick et al., 1993, Proc. Natl. Acad. Sci. USA 90: 4591-4595). Expression of IGF-I (insulin-like growth factor-I) was studied after balloon deendothelialization of rat aorta (Cercek et al., 1990, Circulation Research 66: 1755-1760).

Other studies have focused on the expression of adhesion-molecules on the surface of activated endothelial cells which mediate monocyte adhesion. These adhesion

molecules include intracellular adhesion molecule-1, ICAM-1 (Simmons et al., 1988, Nature, 331: 624-627), ELAM (Bevilacqua et al., 1989, Science 243: 1160-1165; Bevilacqua et al., 1991, Cell 67: 233), and vascular cell adhesion molecule, VCAM-1 (Osborn et al., 1989, Cell 59: 1203-1211); all of these surface molecules are induced transcriptionally in the presence of IL-1. Histological studies reveal that ICAM-1, ELAM and VCAM-1 are expressed on endothelial cells in areas of lesion formation in vivo (Cybulsky et al., 1991, Science 251: 788-791; 1991, Arterioscler. Thromb. 11: 1397a; Poston et al., 1992, Am. J. Pathol. 140: 665-673). VCAM-1 and ICAM-1 were shown to be induced in cultured rabbit arterial endothelium, as well as in cultured human iliac artery endothelial cells by lysophosphatidylcholine, a major phospholipid component of atherogenic lipoproteins. (Kume et al., 1992, J. Clin. Invest. 90: 1138-1144). VCAM-I, ICAM-1, and class II major histocompatibility antigens were reported to be induced in response to injury to rabbit aorta (Tanaka, et al., 1993, Circulation 88: 1788-1803).

20 Cytomegalovirus (CMV) has been implicated in restenosis as well as atherosclerosis in general (Speir, et al., 1994, Science 265: 391-394). It was observed that the CMV protein IE84 apparently predisposes smooth muscle cells to increased growth at the site of restenosis by combining with and inactivating p53 protein, which is known to suppress tumors in its active form.

The foregoing studies are aimed at defining the role of particular gene products presumed to be involved in the excessive inflammatory-fibroproliferative response leading to atherosclerotic plaque formation. However, such approaches cannot identify the full panoply of gene products that are involved in the disease process, much less identify those which may serve as therapeutic targets for the diagnosis and treatment of various forms of cardiovascular disease.

3. SUMMARY OF THE INVENTION

The present invention relates to methods and compositions for the treatment and diagnosis of cardiovascular disease, including but not limited to, atherosclerosis, ischemia/reperfusion, hypertension, restenosis, and arterial inflammation. Specifically, genes are identified and described which are differentially expressed in cardiovascular disease states, relative to their expression in normal, or non-cardiovascular disease states.

"Differential expression", as used herein, refers to both quantitative as well as qualitative differences in the genes' temporal and/or tissue expression patterns. Differentially expressed genes may represent "fingerprint genes," and/or "target genes." "Fingerprint gene," as used herein, refers to a differentially expressed gene whose expression pattern may be utilized as part of a prognostic or diagnostic cardiovascular disease evaluation, or which, alternatively, may be used in methods for identifying compounds useful for the treatment of cardiovascular disease.

"Target gene", as used herein, refers to a differentially expressed gene involved in cardiovascular disease such that modulation of the level of target gene expression or of target gene product activity may act to ameliorate a cardiovascular disease condition. Compounds that modulate target gene expression or activity of the target gene product can be used in the treatment of cardiovascular disease.

Further, "pathway genes" are defined via the ability of their products to interact with other gene products involved in cardiovascular disease. Pathway genes may also exhibit target gene and/or fingerprint gene characteristics. Although the genes described herein may be differentially expressed with respect to cardiovascular disease, and/or their products may interact with gene products important to cardiovascular disease, the genes may also be involved in mechanisms important to additional cardiovascular processes.

The invention includes the products of such fingerprint, target, and pathway genes, as well as antibodies to such gene products. Furthermore, the engineering and use of cell- and animal-based models of cardiovascular disease to which such gene products may contribute are also described.

The present invention encompasses methods for prognostic and diagnostic evaluation of cardiovascular disease conditions, and for the identification of subjects exhibiting a predisposition to such conditions. Furthermore, the invention provides methods for evaluating the efficacy of drugs, and monitoring the progress of patients, involved in clinical trials for the treatment of cardiovascular disease.

The invention also provides methods for the identification of compounds that modulate the expression of genes or the activity of gene products involved in cardiovascular disease, as well as methods for the treatment of cardiovascular disease which may involve the administration of such compounds to individuals exhibiting cardiovascular disease symptoms or tendencies.

The invention is based, in part, on systematic search strategies involving in vivo and in vitro cardiovascular disease paradigms coupled with sensitive and high throughput gene expression assays. In contrast to approaches that merely evaluate the expression of a given gene product presumed to play a role in a disease process, the search strategies and assays used herein permit the identification of all genes, whether known or novel, that are expressed or repressed in the disease condition, as well as the evaluation of their temporal regulation and function during disease progression. This comprehensive approach and evaluation permits the discovery of novel genes and gene products, as well as the identification of an array of genes and gene products (whether novel or known) involved in novel pathways that play a major role in the disease pathology. Thus, the invention allows one to define targets useful for diagnosis, monitoring, rational drug screening and design, and/or other therapeutic intervention.

In the working examples described herein, five novel human genes are identified that are demonstrated to be differentially expressed in different cardiovascular disease states. The identification of these genes and the
5 characterization of their expression in particular disease states provide newly identified roles in cardiovascular disease for these genes.

Specifically, fchd531, fchd540, and fchd545 are novel genes that are each differentially regulated in
10 endothelial cells subjected to shear stress. fchd531 and fchd545 are each down-regulated, whereas fchd540 is up-regulated by shear stress. fchd602 and fchd605 are novel genes that are each up-regulated in monocytes treated with oxidized LDL. Accordingly, methods are provided for the
15 diagnosis, monitoring in clinical trials, screening for therapeutically effective compounds, and treatment of cardiovascular disease based upon the discoveries herein regarding the expression patterns of fchd531, fchd540, fchd545, fchd602, and fchd605.

20 The characteristic up-regulation of genes fchd540, fchd602, and fchd605 can be used to design cardiovascular disease treatment strategies. For those up-regulated genes that have a causative effect on the disease conditions, treatment methods can be designed to reduce or eliminate
25 their expression, particularly in endothelial cells or monocytes. Alternatively, treatment methods include inhibiting the activity of the protein products of these genes. For those up-regulated genes that have a protective effect, treatment methods can be designed for enhancing the
30 activity of the products of such genes.

In either situation, detecting expression of these genes in excess of normal expression provides for the diagnosis of cardiovascular disease. Furthermore, in testing the efficacy of compounds during clinical trials, a decrease
35 in the level of the expression of these genes corresponds to a return from a disease condition to a normal state, and thereby indicates a positive effect of the compound. The

cardiovascular diseases that may be so diagnosed, monitored in clinical trials, and treated include but are not limited to atherosclerosis, ischemia/reperfusion, hypertension, restenosis, and arterial inflammation.

5 The characteristic down-regulation of fchd531 and fchd545 can also be used to design cardiovascular disease treatment strategies. For those genes whose down-regulation has a pathogenic effect, treatment methods can be designed to restore or increase their expression, particularly in
10 endothelial cells. Alternatively, treatment methods include increasing the activity of the protein products of these genes. For those genes whose down-regulation has a protective effect, treatment methods can be designed for decreasing the amount or activity of the products of such
15 genes.

 In either situation, detecting expression of these genes in below normal expression provides for the diagnosis of cardiovascular disease. Furthermore, in testing the efficacy of compounds during clinical trials, an increase in
20 the level of the expression of these genes corresponds to a return from a disease condition to a normal state, and thereby indicates a positive effect of the compound. The cardiovascular diseases that may be so diagnosed, monitored in clinical trials, and treated include but are not limited
25 to atherosclerosis, ischemia/reperfusion, hypertension, restenosis, and arterial inflammation.

 The invention encompasses methods for screening compounds and other substances for treating cardiovascular disease by assaying their ability to modulate the expression
30 of the target genes disclosed herein or activity of the protein products of the target genes. Such screening methods include, but are not limited to, assays for identifying compounds and other substances that interact with (e.g., bind to) the target gene protein products.

35 In addition, the invention encompasses methods for treating cardiovascular disease by administering compounds and other substances that modulate the overall activity of

the target gene products. Compounds and other substances can effect such modulation either on the level of target gene expression or target protein activity.

The invention is based in part on the
5 identification of novel protein-protein interactions of the rchd534 protein with itself and with the fchd540 protein, as well as interactions of the rchd534 protein or the fchd540 protein with other protein members of the TGF- β signalling pathway. The rchd534 gene was described in Applicant's co-
10 pending PCT Application Publication No. WO 96/24604, which is hereby incorporated by reference in its entirety. Screening methods are provided for identifying compounds and other substances for treating cardiovascular disease by assaying their ability to inhibit these interactions. Furthermore,
15 methods are provided for identifying compounds and other substances that enhance the TGF- β response by modulating the activity of the expression of the rchd534 or fchd540 genes or the activity of their gene products. In addition, methods are provided for treating cardiovascular disease by
20 administering compounds and other substances that inhibit these protein interactions.

The invention is based in part on the
identification of the endothelial cell specific expression pattern of two genes, rchd534 and rchd540, whose protein
25 products inhibit the TGF- β response. Accordingly, the rchd534 and rchd540 genes may be targets for intervention in a variety of inflammatory and fibroproliferative disorders that involve endothelial cells, including, but not limited to, cancer angiogenesis, inflammation, and fibrosis.

30 Membrane bound target gene products containing extracellular domains can be a particularly useful target for treatment methods as well as diagnostic and clinical monitoring methods. The fchd602 gene, for example, encodes a transmembrane protein, which contains multiple transmembrane
35 domains and, therefore, can be readily contacted by other compounds on the cell surface. Accordingly, natural ligands, derivatives of natural ligands, and antibodies that bind to

the fchd602 gene product can be utilized to inhibit its activity, or alternatively, to target the specific destruction of cells that express the gene. Furthermore, the extracellular domains of the fchd602 gene product provide
5 targets which allow for the design of especially efficient screening systems for identifying compounds that bind to the fchd602 gene product.

Such an assay system can also be used to screen and identify antagonists of the interaction between the fchd602
10 gene product and ligands that bind to the fchd602 gene product. For example, the compounds can act as decoys by binding to the endogenous (i.e., natural) ligand for the fchd602 gene product. The resulting reduction in the amount of ligand-bound fchd602 gene transmembrane protein will
15 modulate the activity of disease state cells, such as monocytes. Soluble proteins or peptides, such as peptides comprising one or more of the extracellular domains, or portions and/or analogs thereof of the fchd602 gene product, including, for example, soluble fusion proteins such as Ig-
20 tailed fusion proteins, can be particularly useful for this purpose.

Similarly, antibodies that are specific to one or more of the extracellular domains of the fchd602 product provide for the ready detection of this target gene product
25 in diagnostic tests or in clinical test monitoring. Accordingly, endothelial cells can be treated, either in vivo or in vitro, with such a labeled antibody to determine the disease state of endothelial cells. Because the fchd602 gene product is up-regulated in monocytes in the disease state,
30 its detection positively corresponds with cardiovascular disease.

Such methods for treatment, diagnosis, and clinical test monitoring which use the fchd602 gene product as described above can also be applied to other target genes
35 that encode transmembrane gene products, including but not limited to the fchd545 gene, which encodes multiple transmembrane domains and extracellular domains.

The examples presented in Sections 6 and 7, below, demonstrate the use of the cardiovascular disease paradigms of the invention to identify cardiovascular disease target genes.

- 5 The example presented in Section 8, below, demonstrates the use of fingerprint genes in diagnostics and as surrogate markers for testing the efficacy of candidate drugs in basic research and in clinical trials.

 The example presented in Section 9, below,
10 demonstrates the use of fingerprint genes, particularly fchd545, in the imaging of a diseased cardiovascular tissue.

 The example presented in Section 11, below, demonstrates the interaction of two target gene products, the rchd534 and fchd540 proteins, and the further
15 characterization of their roles in cardiovascular disease and the TGF- β signalling pathway.

4. DESCRIPTION OF THE FIGURES

 FIG.1. Nucleotide sequence and encoded amino acid
20 sequence of the fchd531 gene.

 FIG.2. Nucleotide sequence and encoded amino acid sequence of the fchd540 gene.

 FIG.3. Nucleotide sequence and encoded amino acid sequence of the fchd545 gene.

25 FIG.4. Nucleotide sequence and encoded amino acid sequence from the fchd602 gene.

 FIG.5. Nucleotide sequence and encoded amino acid sequence from the fchd605 gene.

 FIG.6. Nucleotide sequence and encoded amino acid
30 sequence of the rchd534 gene.

5. DETAILED DESCRIPTION OF THE INVENTION

 Methods and compositions for the diagnosis and treatment of cardiovascular disease, including but not
35 limited to atherosclerosis, ischemia/reperfusion, hypertension, restenosis, and arterial inflammation, are described. The invention is based, in part, on the

evaluation of the expression and role of all genes that are differentially expressed in paradigms that are physiologically relevant to the disease condition. This permits the definition of disease pathways and the
5 identification of targets in the pathway that are useful both diagnostically and therapeutically.

Genes, termed "target genes" and/or "fingerprint genes" which are differentially expressed in cardiovascular disease conditions, relative to their expression in normal,
10 or non-cardiovascular disease conditions, are described in Section 5.4. Additionally, genes, termed "pathway genes" whose gene products exhibit an ability to interact with gene products involved in cardiovascular disease are also described in Section 5.4. Pathway genes may additionally
15 have fingerprint and/or target gene characteristics. Methods for the identification of such fingerprint, target, and pathway genes are described in Sections 5.1, 5.2, and 5.3.

Further, the gene products of such fingerprint, target, and pathway genes are described in Section 5.4.2,
20 antibodies to such gene products are described in Section 5.4.3, as are cell- and animal-based models of cardiovascular disease to which such gene products may contribute, in Section 5.4.4.

Methods for the identification of compounds which
25 modulate the expression of genes or the activity of gene products involved in cardiovascular disease are described in Section 5.5. Methods for monitoring the efficacy of compounds during clinical trials are described in Section 5.5.4. Additionally described below, in Section 5.6, are
30 methods for the treatment of cardiovascular disease.

Also discussed below, in Section 5.8, are methods for prognostic and diagnostic evaluation of cardiovascular disease, including the identification of subjects exhibiting a predisposition to this disease, and the imaging of
35 cardiovascular disease conditions.

5.1. IDENTIFICATION OF DIFFERENTIALLY EXPRESSED GENES

This section describes methods for the identification of genes which are involved in cardiovascular disease, including but not limited to atherosclerosis, ischemia/reperfusion, hypertension, restenosis, and arterial inflammation. Such genes may represent genes which are differentially expressed in cardiovascular disease conditions relative to their expression in normal, or non-cardiovascular disease conditions. Such differentially expressed genes may represent "target" and/or "fingerprint" genes. Methods for the identification of such differentially expressed genes are described, below, in this section. Methods for the further characterization of such differentially expressed genes, and for their identification as target and/or fingerprint genes, are presented, below, in Section 5.3.

"Differential expression" as used herein refers to both quantitative as well as qualitative differences in the genes' temporal and/or tissue expression patterns. Thus, a differentially expressed gene may have its expression activated or completely inactivated in normal versus cardiovascular disease conditions (e.g., treated with oxidized LDL versus untreated), or under control versus experimental conditions. Such a qualitatively regulated gene will exhibit an expression pattern within a given tissue or cell type which is detectable in either control or cardiovascular disease subjects, but is not detectable in both. Alternatively, such a qualitatively regulated gene will exhibit an expression pattern within a given tissue or cell type which is detectable in either control or experimental subjects, but is not detectable in both.

"Detectable", as used herein, refers to an RNA expression pattern which is detectable via the standard techniques of differential display, reverse transcriptase- (RT-) PCR and/or Northern analyses, which are well known to those of skill in the art.

Alternatively, a differentially expressed gene may have its expression modulated, i.e., quantitatively increased

or decreased, in normal versus cardiovascular disease states, or under control versus experimental conditions. The degree to which expression differs in normal versus cardiovascular disease or control versus experimental states need only be
5 large enough to be visualized via standard characterization techniques, such as, for example, the differential display technique described below. Other such standard characterization techniques by which expression differences may be visualized include but are not limited to quantitative
10 RT-PCR and Northern analyses.

Differentially expressed genes may be further described as target genes and/or fingerprint genes. "Fingerprint gene," as used herein, refers to a differentially expressed gene whose expression pattern may be
15 utilized as part of a prognostic or diagnostic cardiovascular disease evaluation, or which, alternatively, may be used in methods for identifying compounds useful for the treatment of cardiovascular disease. A fingerprint gene may also have the characteristics of a target gene.

20 "Target gene", as used herein, refers to a differentially expressed gene involved in cardiovascular disease in a manner by which modulation of the level of target gene expression or of target gene product activity may act to ameliorate symptoms of cardiovascular disease. A
25 target gene may also have the characteristics of a fingerprint gene.

A variety of methods may be utilized for the identification of genes which are involved in cardiovascular disease. These methods include but are not limited to the
30 experimental paradigms described, below, in Section 5.1.1. Material from the paradigms may be characterized for the presence of differentially expressed gene sequences as discussed, below, in Section 5.1.2.

5.1.1. PARADIGMS FOR THE IDENTIFICATION OF DIFFERENTIALLY EXPRESSED GENES

One strategy for identifying genes that are involved in cardiovascular disease is to detect genes that are expressed differentially under conditions associated with the disease versus non-disease conditions. The sub-sections below describe a number of experimental systems, called paradigms, which may be used to detect such differentially expressed genes. In general, the paradigms include at least one experimental condition in which subjects or samples are treated in a manner associated with cardiovascular disease, in addition to at least one experimental control condition lacking such disease associated treatment. Differentially expressed genes are detected, as described herein, below, by comparing the pattern of gene expression between the experimental and control conditions.

Once a particular gene has been identified through the use of one such paradigm, its expression pattern may be further characterized by studying its expression in a different paradigm. A gene may, for example, be regulated one way in a given paradigm (e.g., up-regulation), but may be regulated differently in some other paradigm (e.g., down-regulation). Furthermore, while different genes may have similar expression patterns in one paradigm, their respective expression patterns may differ from one another under a different paradigm. Such use of multiple paradigms may be useful in distinguishing the roles and relative importance of particular genes in cardiovascular disease.

30 5.1.1.1. FOAM CELL PARADIGM - 1

Among the paradigms which may be utilized for the identification of differentially expressed genes involved in atherosclerosis, for example, are paradigms designed to analyze those genes which may be involved in foam cell formation. Such paradigms may serve to identify genes involved in the differentiation of this cell type, or their uptake of oxidized LDL.

One embodiment of such a paradigm, hereinafter referred to as Paradigm A, is carried out as follows: First, human blood is drawn and peripheral monocytes are isolated by methods routinely practiced in the art. These human
5 monocytes can then be used immediately or cultured in vitro, using methods routinely practiced in the art, for 5 to 9 days where they develop more macrophage-like characteristics such as the up-regulation of scavenger receptors. These cells are then treated for various lengths of time with agents thought
10 to be involved in foam cell formation. These agents include but are not limited to oxidized LDL, acetylated LDL, lysophosphatidylcholine, and homocysteine. Control monocytes that are untreated or treated with native LDL are grown in parallel. At a certain time after addition of the test
15 agents, the cells are harvested and analyzed for differential expression as described in detail in Section 5.1.2., below. The Example presented in Section 6, below, demonstrates in detail the use of such a foam cell paradigm to identify genes which are differentially expressed in treated versus control
20 cells.

5.1.1.2. FOAM CELL PARADIGM - 2

Alternative paradigms involving monocytes for detecting differentially expressed genes associated with
25 atherosclerosis involve the simulation of the phenomenon of transmigration. When monocytes encounter arterial injury, they adhere to the vascular endothelial layer, transmigrate across this layer, and locate between the endothelium and the layer of smooth muscle cells that ring the artery. This
30 phenomenon can be mimicked in vitro by culturing a layer of endothelial cells isolated, for example, from human umbilical cord. Once the endothelial monolayer forms, monocytes drawn from peripheral blood are cultured on top of the endothelium in the presence and absence of LDL. After several hours, the
35 monocytes transmigrate through the endothelium and develop into foam cells after 3 to 5 days when exposed to LDL. In this system, as in vivo, the endothelial cells carry out the

oxidation of LDL which is then taken up by the monocytes. As described in sub-section 5.1.2. below, the pattern of gene expression can then be compared between these foam cells and untreated monocytes.

5

5.1.1.3. FOAM CELL PARADIGM - 3

Yet another system includes the third cell type, smooth muscle cell, that plays a critical role in atherogenesis (Navab et al., 1988, J. Clin. Invest., 82: 1853). In this system, a multilayer of human aortic smooth muscle cells was grown on a micropore filter covered with a gel layer of native collagen, and a monolayer of human aortic endothelial cells was grown on top of the collagen layer. Exposure of this coculture to human monocytes in the presence of chemotactic factor rFMLP resulted in monocyte attachment to the endothelial cells followed by migration across the endothelial monolayer into the collagen layer of the subendothelial space. This type of culture can also be treated with LDL to generate foam cells. The foam cells can then be harvested and their pattern of gene expression compared to that of untreated cells as explained below in sub-section 5.1.2.

5.1.1.4. IN VIVO MONOCYTE PARADIGM

An alternative embodiment of such paradigms for the study of monocytes, hereinafter referred to as Paradigm B, involves differential treatment of human subjects through the dietary control of lipid consumption. Such human subjects are held on a low fat/low cholesterol diet for three weeks, at which time blood is drawn, monocytes are isolated according to the methods routinely practiced in the art, and RNA is purified, as described below, in sub-section 5.1.2. These same patients are subsequently switched to a high fat/high cholesterol diet and monocyte RNA is purified again. The patients may also be fed a third, combination diet containing high fat/low cholesterol and monocyte RNA may be purified once again. The order in which patients receive the

diets may be varied. The RNA derived from patients maintained on two of the diets, or on all three diets, may then be compared and analyzed for differential gene expression as, explained below in sub-section 5.1.2.

5

5.1.1.5. ENDOTHELIAL CELL - IL-1 PARADIGM

In addition to the detection of differential gene expression in monocytes, paradigms focusing on endothelial cells may be used to detect genes involved in cardiovascular
10 disease. In one such paradigm, hereinafter referred to as Paradigm C, human umbilical vein endothelial cells (HUVEC's) are grown in vitro. Experimental cultures are treated with human IL-1 β , a factor known to be involved in the inflammatory response, in order to mimic the physiologic
15 conditions involved in the atherosclerotic state. Alternatively experimental HUVEC cultures may be treated with lysophosphatidylcholine, a major phospholipid component of atherogenic lipoproteins or oxidized human LDL. Control cultures are grown in the absence of these compounds.
20 After a certain period of treatment, experimental and control cells are harvested and analyzed for differential gene expression as described in sub-section 5.1.2, below.

25

5.1.1.6. ENDOTHELIAL CELL - SHEAR STRESS PARADIGM

In another paradigm involving endothelial cells, hereinafter referred to as Paradigm D, cultures are exposed to fluid shear stress which is thought to be responsible for
30 the prevalence of atherosclerotic lesions in areas of unusual circulatory flow. Unusual blood flow also plays a role in the harmful effects of ischemia/reperfusion, wherein an organ receiving inadequate blood supply is suddenly reperfused with an overabundance of blood when the obstruction is overcome.

35

Cultured HUVEC monolayers are exposed to laminar sheer stress by rotating the culture in a specialized apparatus containing liquid culture medium (Nagel et al.,

1994, J. Clin. Invest. 94: 885-891). Static cultures grown in the same medium serve as controls. After a certain period of exposure to shear stress, experimental and control cells are harvested and analyzed for differential gene expression as described in sub-section 5.1.2, below. The Example presented in Section 7, below, demonstrates the use of such a shear stressed endothelial cell paradigm to identify sequences which are differentially expressed in exposed versus control cells.

10 In all such paradigms designed to identify genes which are involved in cardiovascular disease, including but not limited to those described above in Sections 5.1.1.1 through 5.1.1.6, compounds such as drugs known to have an ameliorative effect on the disease symptoms may be
15 incorporated into the experimental system. Such compounds may include known therapeutics, as well as compounds that are not useful as therapeutics due to their harmful side effects. Test cells that are cultured as explained in the paradigms described in Sections 5.1.1.1 through 5.1.1.6, for example,
20 may be exposed to one of these compounds and analyzed for differential gene expression with respect to untreated cells, according to the methods described below in Section 5.1.2. In principle, according to the particular paradigm, any cell type involved in the disease may be treated at any stage of
25 the disease process by these compounds.

Test cells may also be compared to unrelated cells (e.g., fibroblasts) that are also treated with the compound, in order to screen out generic effects on gene expression that might not be related to the disease. Such generic
30 effects might be manifest by changes in gene expression that are common to the test cells and the unrelated cells upon treatment with the compound.

By these methods, the genes and gene products upon which these compounds act can be identified and used in the
35 assays described below to identify novel therapeutic compounds for the treatment of cardiovascular disease.

5.1.2. ANALYSIS OF PARADIGM MATERIAL

In order to identify differentially expressed genes, RNA, either total or mRNA, may be isolated from one or more tissues of the subjects utilized in paradigms such as those described earlier in this Section. RNA samples are obtained from tissues of experimental subjects and from corresponding tissues of control subjects. Any RNA isolation technique which does not select against the isolation of mRNA may be utilized for the purification of such RNA samples.

See, for example, Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, N.Y.; and Ausubel, F.M. et al., eds., 1987-1993, Current Protocols in Molecular Biology, John Wiley & Sons, Inc. New York, both of which are incorporated herein by reference in their entirety.

Additionally, large numbers of tissue samples may readily be processed using techniques well known to those of skill in the art, such as, for example, the single-step RNA isolation process of Chomczynski, P. (1989, U.S. Patent No. 4,843,155), which is incorporated herein by reference in its entirety.

Transcripts within the collected RNA samples which represent RNA produced by differentially expressed genes may be identified by utilizing a variety of methods which are well known to those of skill in the art. For example, differential screening (Tedder, T.F. et al., 1988, Proc. Natl. Acad. Sci. USA 85:208-212), subtractive hybridization (Hedrick, S.M. et al., 1984, Nature 308:149-153; Lee, S.W. et al., 1984, Proc. Natl. Acad. Sci. USA 88:2825), and, preferably, differential display (Liang, P., and Pardee, A.B., 1993, U.S. Patent No. 5,262,311, which is incorporated herein by reference in its entirety), may be utilized to identify nucleic acid sequences derived from genes that are differentially expressed.

Differential screening involves the duplicate screening of a cDNA library in which one copy of the library is screened with a total cell cDNA probe corresponding to the mRNA population of one cell type while a duplicate copy of the cDNA library is screened with a total cDNA probe

corresponding to the mRNA population of a second cell type. For example, one cDNA probe may correspond to a total cell cDNA probe of a cell type derived from a control subject, while the second cDNA probe may correspond to a total cell
5 cDNA probe of the same cell type derived from an experimental subject. Those clones which hybridize to one probe but not to the other potentially represent clones derived from genes differentially expressed in the cell type of interest in control versus experimental subjects.

10 Subtractive hybridization techniques generally involve the isolation of mRNA taken from two different sources, e.g., control and experimental tissue, the hybridization of the mRNA or single-stranded cDNA reverse-transcribed from the isolated mRNA, and the removal of all
15 hybridized, and therefore double-stranded, sequences. The remaining non-hybridized, single-stranded cDNAs, potentially represent clones derived from genes that are differentially expressed in the two mRNA sources. Such single-stranded cDNAs are then used as the starting material for the
20 construction of a library comprising clones derived from differentially expressed genes.

The differential display technique describes a procedure, utilizing the well known polymerase chain reaction (PCR; the experimental embodiment set forth in Mullis, K.B.,
25 1987, U.S. Patent No. 4,683,202) which allows for the identification of sequences derived from genes which are differentially expressed. First, isolated RNA is reverse-transcribed into single-stranded cDNA, utilizing standard techniques which are well known to those of skill in the art.
30 Primers for the reverse transcriptase reaction may include, but are not limited to, oligo dT-containing primers, preferably of the reverse primer type of oligonucleotide described below. Next, this technique uses pairs of PCR primers, as described below, which allow for the
35 amplification of clones representing a random subset of the RNA transcripts present within any given cell. Utilizing different pairs of primers allows each of the mRNA

transcripts present in a cell to be amplified. Among such amplified transcripts may be identified those which have been produced from differentially expressed genes.

The reverse oligonucleotide primer of the primer
5 pairs may contain an oligo dT stretch of nucleotides, preferably eleven nucleotides long, at its 5' end, which hybridizes to the poly(A) tail of mRNA or to the complement of a cDNA reverse transcribed from an mRNA poly(A) tail. Second, in order to increase the specificity of the reverse
10 primer, the primer may contain one or more, preferably two, additional nucleotides at its 3' end. Because, statistically, only a subset of the mRNA derived sequences present in the sample of interest will hybridize to such primers, the additional nucleotides allow the primers to
15 amplify only a subset of the mRNA derived sequences present in the sample of interest. This is preferred in that it allows more accurate and complete visualization and characterization of each of the bands representing amplified sequences.

20 The forward primer may contain a nucleotide sequence expected, statistically, to have the ability to hybridize to cDNA sequences derived from the tissues of interest. The nucleotide sequence may be an arbitrary one, and the length of the forward oligonucleotide primer may
25 range from about 9 to about 13 nucleotides, with about 10 nucleotides being preferred. Arbitrary primer sequences cause the lengths of the amplified partial cDNAs produced to be variable, thus allowing different clones to be separated by using standard denaturing sequencing gel electrophoresis.

30 PCR reaction conditions should be chosen which optimize amplified product yield and specificity, and, additionally, produce amplified products of lengths which may be resolved utilizing standard gel electrophoresis techniques. Such reaction conditions are well known to those
35 of skill in the art, and important reaction parameters include, for example, length and nucleotide sequence of

oligonucleotide primers as discussed above, and annealing and elongation step temperatures and reaction times.

The pattern of clones resulting from the reverse transcription and amplification of the mRNA of two different
5 cell types is displayed via sequencing gel electrophoresis and compared. Differences in the two banding patterns indicate potentially differentially expressed genes.

Once potentially differentially expressed gene sequences have been identified via bulk techniques such as,
10 for example, those described above, the differential expression of such putatively differentially expressed genes should be corroborated. Corroboration may be accomplished via, for example, such well known techniques as Northern analysis and/or RT-PCR.

15 Upon corroboration, the differentially expressed genes may be further characterized, and may be identified as target and/or fingerprint genes, as discussed, below, in Section 5.3.

Also, amplified sequences of differentially
20 expressed genes obtained through, for example, differential display may be used to isolate full length clones of the corresponding gene. The full length coding portion of the gene may readily be isolated, without undue experimentation, by molecular biological techniques well known in the art.
25 For example, the isolated differentially expressed amplified fragment may be labeled and used to screen a cDNA library. Alternatively, the labeled fragment may be used to screen a genomic library.

PCR technology may also be utilized to isolate full
30 length cDNA sequences. As described, above, in this Section, the isolated, amplified gene fragments obtained through differential display have 5' terminal ends at some random point within the gene and have 3' terminal ends at a position preferably corresponding to the 3' end of the transcribed
35 portion of the gene. Once nucleotide sequence information from an amplified fragment is obtained, the remainder of the gene (i.e., the 5' end of the gene, when utilizing

differential display) may be obtained using, for example, RT-PCR.

In one embodiment of such a procedure for the identification and cloning of full length gene sequences, RNA may be isolated, following standard procedures, from an appropriate tissue or cellular source. A reverse transcription reaction may then be performed on the RNA using an oligonucleotide primer complimentary to the mRNA that corresponds to the amplified fragment, for the priming of first strand synthesis. Because the primer is anti-parallel to the mRNA, extension will proceed toward the 5' end of the mRNA. The resulting RNA/DNA hybrid may then be "tailed" with guanines using a standard terminal transferase reaction, the hybrid may be digested with RNAase H, and second strand synthesis may then be primed with a poly-C primer. Using the two primers, the 5' portion of the gene is amplified using PCR. Sequences obtained may then be isolated and recombined with previously isolated sequences to generate a full-length cDNA of the differentially expressed genes of the invention. For a review of cloning strategies and recombinant DNA techniques, see e.g., Sambrook et al., 1989, *supra*; and Ausubel et al., 1989, *supra*.

5.2. IDENTIFICATION OF PATHWAY GENES

This section describes methods for the identification of genes, termed "pathway genes", involved in cardiovascular disease. "Pathway gene", as used herein, refers to a gene whose gene product exhibits the ability to interact with gene products involved in cardiovascular disease. A pathway gene may be differentially expressed and, therefore, may additionally have the characteristics of a target and/or fingerprint gene.

Any method suitable for detecting protein-protein interactions may be employed for identifying pathway gene products by identifying interactions between gene products and gene products known to be involved in cardiovascular disease. Such known gene products may be cellular or

extracellular proteins. Those gene products which interact with such known gene products represent pathway gene products and the genes which encode them represent pathway genes.

Among the traditional methods which may be employed
5 are co-immunoprecipitation, crosslinking and co-purification through gradients or chromatographic columns. Utilizing procedures such as these allows for the identification of pathway gene products. Once identified, a pathway gene product may be used, in conjunction with standard techniques,
10 to identify its corresponding pathway gene. For example, at least a portion of the amino acid sequence of the pathway gene product may be ascertained using techniques well known to those of skill in the art, such as via the Edman degradation technique (see, e.g., Creighton, 1983, Proteins:
15 Structures and Molecular Principles, W.H. Freeman & Co., N.Y., pp.34-49). The amino acid sequence obtained may be used as a guide for the generation of oligonucleotide mixtures that can be used to screen for pathway gene sequences. Screening may be accomplished, for example by
20 standard hybridization or PCR techniques. Techniques for the generation of oligonucleotide mixtures and screening are well-known. (See, e.g., Ausubel, *supra.*, and PCR Protocols: A Guide to Methods and Applications, 1990, Innis, M. et al., eds. Academic Press, Inc., New York).

25 Additionally, methods may be employed which result in the simultaneous identification of pathway genes which encode the protein interacting with a protein involved in cardiovascular disease. These methods include, for example, probing expression libraries with labeled protein known or
30 suggested to be involved in cardiovascular disease, using this protein in a manner similar to the well known technique of antibody probing of λ gt11 libraries.

One such method which detects protein interactions *in vivo*, the two-hybrid system, is described in detail for
35 illustration only and not by way of limitation. One version of this system has been described (Chien et al., 1991, Proc.

Natl. Acad. Sci. USA, 88:9578-9582) and is commercially available from Clontech (Palo Alto, CA).

Briefly, utilizing such a system, plasmids are constructed that encode two hybrid proteins: one consists of the DNA-binding domain of a transcription activator protein fused to a known protein, and the other consists of the activator protein's activation domain fused to an unknown protein that is encoded by a cDNA which has been recombined into this plasmid as part of a cDNA library. The plasmids are transformed into a strain of the yeast Saccharomyces cerevisiae that contains a reporter gene (e.g., lacZ) whose regulatory region contains the activator's binding sites. Either hybrid protein alone cannot activate transcription of the reporter gene; the DNA-binding domain hybrid, because it does not provide activation function and the activation domain hybrid, because it cannot localize to the activator's binding sites. Interaction of the two proteins reconstitutes the functional activator protein and results in expression of the reporter gene, which is detected by an assay for the reporter gene product.

The two-hybrid system or related methodology may be used to screen activation domain libraries for proteins that interact with a known "bait" gene protein. Total genomic or cDNA sequences may be fused to the DNA encoding an activation domain. Such a library and a plasmid encoding a hybrid of the bait gene protein fused to the DNA-binding domain may be cotransformed into a yeast reporter strain, and the resulting transformants may be screened for those that express the reporter gene. These colonies may be purified and the library plasmids responsible for reporter gene expression may be isolated. DNA sequencing may then be used to identify the proteins encoded by the library plasmids.

For example, and not by way of limitation, the bait gene may be cloned into a vector such that it is translationally fused to the DNA encoding the DNA-binding domain of the GAL4 protein. Also by way of example, for the isolation of genes involved in cardiovascular disease,

previously isolated genes known or suggested to play a part in cardiovascular disease may be used as the bait genes. These include but are not limited to the genes for bFGF, IGF-I, VEGF, IL-1, M-CSF, TGF β , TGF α , TNF α , HB-EGF, PDGF, IFN- γ , 5 and GM-CSF, to name a few.

A cDNA library of the cell line from which proteins that interact with bait gene are to be detected can be made using methods routinely practiced in the art. According to the particular system described herein, for example, the cDNA 10 fragments may be inserted into a vector such that they are translationally fused to the activation domain of GAL4. This library may be co-transformed along with the bait gene-GAL4 fusion plasmid into a yeast strain which contains a *lacZ* gene driven by a promoter which contains the GAL4 activation 15 sequence. A cDNA encoded protein, fused to the GAL4 activation domain, that interacts with bait gene will reconstitute an active GAL4 protein and thereby drive expression of the *lacZ* gene. Colonies which express *lacZ* may be detected by their blue color in the presence of X-gal. 20 The cDNA may then be purified from these strains, and used to produce and isolate the bait gene-interacting protein using techniques routinely practiced in the art.

Once a pathway gene has been identified and isolated, it may be further characterized as, for example, 25 discussed below, in Section 5.3.

A preferred embodiment of the use of the yeast two-hybrid system is described in detail in the example in Section 12, below. As described in Section 12, the yeast two-hybrid system was used to detect the interaction between 30 the protein products of two target genes, *rchd534* and *fchd540*.

5.3. CHARACTERIZATION OF DIFFERENTIALLY EXPRESSED AND PATHWAY GENES

35 Differentially expressed genes, such as those identified via the methods discussed, above, in Section 5.1.1, pathway genes, such as those identified via the

methods discussed, above, in Section 5.2, as well as genes identified by alternative means, may be further characterized by utilizing, for example, methods such as those discussed herein. Such genes will be referred to herein as "identified 5 genes".

Analyses such as those described herein will yield information regarding the biological function of the identified genes. An assessment of the biological function of the differentially expressed genes, in addition, will 10 allow for their designation as target and/or fingerprint genes. Specifically, any of the differentially expressed genes whose further characterization indicates that a modulation of the gene's expression or a modulation of the gene product's activity may ameliorate cardiovascular disease 15 will be designated "target genes", as defined, above, in Section 5.1. Such target genes and target gene products, along with those discussed below, will constitute the focus of the compound discovery strategies discussed, below, in Section 5.5.

20 Any of the differentially expressed genes whose further characterization indicates that such modulations may not positively affect cardiovascular disease, but whose expression pattern contributes to a gene expression "fingerprint pattern" correlative of, for example, a 25 cardiovascular disease condition will be designated a "fingerprint gene". "Fingerprint patterns" will be more fully discussed, below, in Section 5.8. It should be noted that each of the target genes may also function as fingerprint genes, as may all or a subset of the pathway 30 genes.

It should further be noted that the pathway genes may also be characterized according to techniques such as those described herein. Those pathway genes which yield information indicating that they are differentially expressed 35 and that modulation of the gene's expression or a modulation of the gene product's activity may ameliorate cardiovascular disease will be also be designated "target genes". Such

target genes and target gene products, along with those discussed above, will constitute the focus of the compound discovery strategies discussed, below, in Section 5.5.

It should be additionally noted that the
5 characterization of one or more of the pathway genes may reveal a lack of differential expression, but evidence that modulation of the gene's activity or expression may, nonetheless, ameliorate cardiovascular disease symptoms. In such cases, these genes and gene products would also be
10 considered a focus of the compound discovery strategies of Section 5.5, below.

In instances wherein a pathway gene's characterization indicates that modulation of gene expression or gene product activity may not positively affect
15 cardiovascular disease, but whose expression is differentially expressed and which contributes to a gene expression fingerprint pattern correlative of, for example, a cardiovascular disease state, such pathway genes may additionally be designated as fingerprint genes.

20 Among the techniques whereby the identified genes may be further characterized, the nucleotide sequence of the identified genes, which may be obtained by utilizing standard techniques well known to those of skill in the art, may be used to further characterize such genes. For example, the
25 sequence of the identified genes may reveal homologies to one or more known sequence motifs which may yield information regarding the biological function of the identified gene product.

Second, an analysis of the tissue distribution of
30 the mRNA produced by the identified genes may be conducted, utilizing standard techniques well known to those of skill in the art. Such techniques may include, for example, Northern analyses and RT-PCR. Such analyses provide information as to whether the identified genes are expressed in tissues
35 expected to contribute to cardiovascular disease. Such analyses may also provide quantitative information regarding steady state mRNA regulation, yielding data concerning which

of the identified genes exhibits a high level of regulation in, preferably, tissues which may be expected to contribute to cardiovascular disease.

Such analyses may also be performed on an isolated
5 cell population of a particular cell type derived from a given tissue. Additionally, standard in situ hybridization techniques may be utilized to provide information regarding which cells within a given tissue express the identified gene. Such analyses may provide information regarding the
10 biological function of an identified gene relative to cardiovascular disease in instances wherein only a subset of the cells within the tissue is thought to be relevant to cardiovascular disease.

Third, the sequences of the identified genes may be
15 used, utilizing standard techniques, to place the genes onto genetic maps, e.g., mouse (Copeland & Jenkins, 1991, Trends in Genetics 7: 113-118) and human genetic maps (Cohen, et al., 1993, Nature 366: 698-701). Such mapping information may yield information regarding the genes' importance to
20 human disease by, for example, identifying genes which map near genetic regions to which known genetic cardiovascular disease tendencies map.

Fourth, the biological function of the identified genes may be more directly assessed by utilizing relevant in
25 vivo and in vitro systems. In vivo systems may include, but are not limited to, animal systems which naturally exhibit cardiovascular disease predisposition, or ones which have been engineered to exhibit such symptoms, including but not limited to the apoE-deficient atherosclerosis mouse model
30 (Plump et al., 1992, Cell 71: 343-353). Such systems are discussed in Section 5.4.4.1, below.

In vitro systems may include, but are not limited to, cell-based systems comprising cell types known or suspected of involvement in cardiovascular disease. Such
35 systems are discussed in detail, below, in Section 5.4.4.2.

In further characterizing the biological function of the identified genes, the expression of these genes may be

modulated within the in vivo and/or in vitro systems, i.e., either over- or underexpressed, and the subsequent effect on the system then assayed. Alternatively, the activity of the product of the identified gene may be modulated by either
5 increasing or decreasing the level of activity in the in vivo and/or in vitro system of interest, and its subsequent effect then assayed.

The information obtained through such characterizations may suggest relevant methods for the
10 treatment of cardiovascular disease involving the gene of interest. For example, treatment may include a modulation of gene expression and/or gene product activity. Characterization procedures such as those described herein may indicate where such modulation should involve an increase
15 or a decrease in the expression or activity of the gene or gene product of interest.

For example, genes which are up-regulated under disease conditions may be involved in causing or exacerbating the disease condition. Treatments directed at down-
20 regulating the activity of such harmfully expressed genes will ameliorate the disease condition. On the other hand, the up-regulation of genes under disease conditions may be part of a protective response by affected cells. Treatments directed at increasing or enhancing the activity of such up-
25 regulated gene products, especially in individuals lacking normal up-regulation, will similarly ameliorate disease conditions. Such methods of treatment are discussed, below, in Section 5.6.

30 5.4. DIFFERENTIALLY EXPRESSED AND PATHWAY GENES

Identified genes, which include but are not limited to differentially expressed genes such as those identified in Section 5.1.1, above, and pathway genes, such as those identified in Section 5.2, above, are described herein.
35 Specifically, the nucleic acid sequences and gene products of such identified genes are described herein. Further, antibodies directed against the identified genes' products,

and cell- and animal-based models by which the identified genes may be further characterized and utilized are also discussed in this Section.

5 5.4.1. DIFFERENTIALLY EXPRESSED AND PATHWAY GENE SEQUENCES

 The differentially expressed and pathway genes of the invention are listed below, in Table 1. Differentially expressed and pathway gene nucleotide sequences are shown in
10 FIGS. 8, 12, 15, 18, 22, 28, 31, and 35.

 Table 1 lists differentially expressed genes identified through, for example, the paradigms discussed, above, in Section 5.1.1, and below, in the examples presented in Sections 6 through 9. Table 1 also summarizes information
15 regarding the further characterization of such genes.

 First, the paradigm used initially to detect the differentially expressed gene is described under the column headed "Paradigm of Original Detection". The expression patterns of those genes which have been shown to be
20 differentially expressed, for example, under one or more of the paradigm conditions described in Section 5.1.1 are summarized under the column headed "Paradigm Expression Pattern". For each of the tested genes, the paradigm which was used and the difference in the expression of the gene
25 among the samples generated is shown. "↑" indicates that gene expression is up-regulated (*i.e.*, there is an increase in the amount of detectable mRNA) among the samples generated, while "↓" indicates that gene expression is down-regulated (*i.e.*, there is a decrease in the amount of
30 detectable mRNA) among the samples generated. "Detectable" as used herein, refers to levels of mRNA which are detectable via, for example, standard Northern and/or RT-PCR techniques which are well known to those of skill in the art.

 Cell types in which differential expression was
35 detected are also summarized in Table 1 under the column headed "Cell Type Detected in". The column headed "Chromosomal Location" provides the human chromosome number

on which the gene is located. Additionally, in instances wherein the genes contain nucleotide sequences similar or homologous to sequences found in nucleic acid databases, references to such similarities are listed.

- 5 The genes listed in Table 1 may be obtained using cloning methods well known to those skilled in the art, including but not limited to the use of appropriate probes to detect the genes within an appropriate cDNA or gDNA (genomic DNA) library. (See, for example, Sambrook et al., 1989, 10 Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratories, which is incorporated by reference herein in its entirety). Probes for the novel sequences reported herein may be obtained directly from the isolated clones deposited with the ATCC, as indicated in Table 2, below.
- 15 Alternatively, oligonucleotide probes for the novel genes may be synthesized based on the DNA sequences disclosed herein in FIGS 1-5.

- The sequence obtained from clones containing partial coding sequences or non-coding sequences can be used 20 to obtain the entire coding region by using the RACE method (Chenchik, et al., 1995, CLONTECHniques (X) 1: 5-8; Barnes, 1994, Proc. Natl. Acad. Sci. USA 91: 2216-2220; and Cheng et al., Proc. Natl. Acad. Sci. USA 91: 5695-5699).
- Oligonucleotides can be designed based on the sequence 25 obtained from the partial clone that can amplify a reverse transcribed mRNA encoding the entire coding sequence.

- Alternatively, probes can be used to screen cDNA libraries prepared from an appropriate cell or cell line in which the gene is transcribed. For example, the genes 30 described herein that were detected in monocytes may be cloned from a cDNA library prepared from monocytes isolated as described in Section 6.1.1, below.

- The genes described herein that were detected in endothelial cells may also be cloned from a cDNA library 35 constructed from endothelial cells isolated as described in Progress in Hemostasis and Thrombosis, Vol. 3, P. Spaet, editor, Grune & Stratton Inc., New York, 1-28.

Alternatively, the genes may be retrieved from a human placenta cDNA library (Clontech Laboratories, Palo Alto, CA), according to Takahashi et al., 1990, supra; a HUVEC cDNA library as described in Jones et al. 1993, supra; or an acute 5 lymphoblastic leukemia (SUP-B2) cDNA library as described in Cleary et al., 1986, supra, for example. Genomic DNA libraries can be prepared from any source.

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TABLE 1
Differentially Expressed and Pathway Genes

Gene	Seq. ID #	Paradigm of Original Detection	Paradigm Expr. Pattern	Cell Type Detected in	Ref	Seq.
fchd531	1	D (Section 5.1.1.1.6)	↓	Endothelial	New, 1	FIG. 1
fchd540	2	D	↑	Endothelial	New, 2	FIG. 2
fchd545	3	D	↓	Endothelial	New, 3	FIG. 3
fchd602	4	A (Section 5.1.1.1.1)	↑	Monocytes	New, 4	FIG. 4
fchd605	5	A	↑	Monocytes	New, 5	FIG. 5

1. GenBank accession number U05343.
2. *Drosophila* Mothers against *app* (*Mad*), Sekelsky et al., 1995, *Genetics* 139: 1347-1358.
3. Human Voltage-dependent Anion Channel, Blachly-Dyson, E., et al., 1993, *J. Biol. Chem.* 268: 1835-1841; and EST T24012
4. Rat CL-6, Diamond, R.H., et al., 1993, *J. Biol. Chem.* 268: 15185-15192.
5. Mouse gly96, Charles, C.H., et al., 1993, *Oncogene* 8: 797-801; and EST T49532.

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Table 2, below, lists the strains of E. coli deposited with the ATCC that contain plasmids bearing the novel genes listed in Table 1.

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TABLE 2

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GENE	Strain Deposited with ATCC
fchd531	pFCHD531
fchd540	pFCHD540
fchd545	fchd545

As used herein, "differentially expressed gene" (i.e. target and fingerprint gene) or "pathway gene" refers to (a) a gene containing at least one of the DNA sequences disclosed herein (as shown in FIGS. 1-5), or contained in the clones listed in Table 2, as deposited with the ATCC; (b) any DNA sequence that encodes the amino acid sequence encoded by the DNA sequences disclosed herein (as shown in FIGS.1-5), contained in the clones, listed in Table 2, as deposited with the ATCC or contained within the coding region of the gene to which the DNA sequences disclosed herein (as shown in FIGS.1-5) or contained in the clones listed in Table 2, as deposited with the ATCC, belong; (c) any DNA sequence that hybridizes to the complement of the coding sequences disclosed herein, contained in the clones listed in Table 2, as deposited with the ATCC, or contained within the coding region of the gene to which the DNA sequences disclosed herein (as shown in FIGS.1-5) or contained in the clones listed in Table 2, as deposited with the ATCC, belong, under highly stringent conditions, e.g., hybridization to filter-bound DNA in 0.5 M NaHPO₄, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65°C, and washing in 0.1xSSC/0.1% SDS at 68°C (Ausubel F.M. et al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York, at p. 2.10.3) and encodes a gene product

functionally equivalent to a gene product encoded by sequences contained within the clones listed in Table 2; and/or (d) any DNA sequence that hybridizes to the complement of the coding sequences disclosed herein, (as shown in 5 FIGS.1-5) contained in the clones listed in Table 2, as deposited with the ATCC or contained within the coding region of the gene to which DNA sequences disclosed herein (as shown in FIGS.1-5) or contained in the clones, listed in Table 2, as deposited with the ATCC, belong, under less stringent 10 conditions, such as moderately stringent conditions, e.g., washing in 0.2xSSC/0.1% SDS at 42°C (Ausubel et al., 1989, *supra*), yet which still encodes a functionally equivalent gene product.

The invention also includes nucleic acid molecules, 15 preferably DNA molecules, that hybridize to, and are therefore the complements of, the DNA sequences (a) through (c), in the preceding paragraph. Such hybridization conditions may be highly stringent or less highly stringent, as described above. In instances wherein the nucleic acid 20 molecules are deoxyoligonucleotides ("oligos"), highly stringent conditions may refer, e.g., to washing in 6xSSC/0.05% sodium pyrophosphate at 37°C (for 14-base oligos), 48°C (for 17-base oligos), 55°C (for 20-base oligos), and 60°C (for 23-base oligos). These nucleic acid 25 molecules may act as target gene antisense molecules, useful, for example, in target gene regulation and/or as antisense primers in amplification reactions of target gene nucleic acid sequences. Further, such sequences may be used as part of ribozyme and/or triple helix sequences, also useful for 30 target gene regulation. Still further, such molecules may be used as components of diagnostic methods whereby the presence of a cardiovascular disease-causing allele, may be detected.

The invention also encompasses (a) DNA vectors that contain any of the foregoing coding sequences and/or their 35 complements (i.e., antisense); (b) DNA expression vectors that contain any of the foregoing coding sequences operatively associated with a regulatory element that directs

the expression of the coding sequences; and (c) genetically engineered host cells that contain any of the foregoing coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences in the host cell. As used herein, regulatory elements include but are not limited to inducible and non-inducible promoters, enhancers, operators and other elements known to those skilled in the art that drive and regulate expression. The invention includes fragments of any of the DNA sequences disclosed herein.

In addition to the gene sequences described above, homologues of such sequences, as may, for example be present in other species, may be identified and may be readily isolated, without undue experimentation, by molecular biological techniques well known in the art. Further, there may exist genes at other genetic loci within the genome that encode proteins which have extensive homology to one or more domains of such gene products. These genes may also be identified via similar techniques.

For example, the isolated differentially expressed gene sequence may be labeled and used to screen a cDNA library constructed from mRNA obtained from the organism of interest. Hybridization conditions will be of a lower stringency when the cDNA library was derived from an organism different from the type of organism from which the labeled sequence was derived. Alternatively, the labeled fragment may be used to screen a genomic library derived from the organism of interest, again, using appropriately stringent conditions. Such low stringency conditions will be well known to those of skill in the art, and will vary predictably depending on the specific organisms from which the library and the labeled sequences are derived. For guidance regarding such conditions see, for example, Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, Cold Springs Harbor Press, N.Y.; and Ausubel et al., 1989, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y.

Further, a previously unknown differentially expressed or pathway gene-type sequence may be isolated by performing PCR using two degenerate oligonucleotide primer pools designed on the basis of amino acid sequences within
5 the gene of interest. The template for the reaction may be cDNA obtained by reverse transcription of mRNA prepared from human or non-human cell lines or tissue known or suspected to express a differentially expressed or pathway gene allele.

The PCR product may be subcloned and sequenced to
10 insure that the amplified sequences represent the sequences of a differentially expressed or pathway gene-like nucleic acid sequence. The PCR fragment may then be used to isolate a full length cDNA clone by a variety of methods. For example, the amplified fragment may be labeled and used to
15 screen a bacteriophage cDNA library. Alternatively, the labeled fragment may be used to screen a genomic library.

PCR technology may also be utilized to isolate full length cDNA sequences. For example, RNA may be isolated, following standard procedures, from an appropriate cellular
20 or tissue source. A reverse transcription reaction may be performed on the RNA using an oligonucleotide primer specific for the most 5' end of the amplified fragment for the priming of first strand synthesis. The resulting RNA/DNA hybrid may then be "tailed" with guanines using a standard terminal
25 transferase reaction, the hybrid may be digested with RNAase H, and second strand synthesis may then be primed with a poly-C primer. Thus, cDNA sequences upstream of the amplified fragment may easily be isolated. For a review of cloning strategies which may be used, see e.g., Sambrook et
30 al., 1989, *supra*.

In cases where the differentially expressed or pathway gene identified is the normal, or wild type, gene, this gene may be used to isolate mutant alleles of the gene. Such an isolation is preferable in processes and disorders
35 which are known or suspected to have a genetic basis. Mutant alleles may be isolated from individuals either known or suspected to have a genotype which contributes to

cardiovascular disease symptoms. Mutant alleles and mutant allele products may then be utilized in the therapeutic and diagnostic assay systems described below.

A cDNA of the mutant gene may be isolated, for example, by using PCR, a technique which is well known to those of skill in the art. In this case, the first cDNA strand may be synthesized by hybridizing an oligo-dT oligonucleotide to mRNA isolated from tissue known or suspected to be expressed in an individual putatively carrying the mutant allele, and by extending the new strand with reverse transcriptase. The second strand of the cDNA is then synthesized using an oligonucleotide that hybridizes specifically to the 5' end of the normal gene. Using these two primers, the product is then amplified via PCR, cloned into a suitable vector, and subjected to DNA sequence analysis through methods well known to those of skill in the art. By comparing the DNA sequence of the mutant gene to that of the normal gene, the mutation(s) responsible for the loss or alteration of function of the mutant gene product can be ascertained.

Alternatively, a genomic or cDNA library can be constructed and screened using DNA or RNA, respectively, from a tissue known to or suspected of expressing the gene of interest in an individual suspected of or known to carry the mutant allele. The normal gene or any suitable fragment thereof may then be labeled and used as a probe to identify the corresponding mutant allele in the library. The clone containing this gene may then be purified through methods routinely practiced in the art, and subjected to sequence analysis as described, above, in this Section.

Additionally, an expression library can be constructed utilizing DNA isolated from or cDNA synthesized from a tissue known to or suspected of expressing the gene of interest in an individual suspected of or known to carry the mutant allele. In this manner, gene products made by the putatively mutant tissue may be expressed and screened using standard antibody screening techniques in conjunction with

antibodies raised against the normal gene product, as described, below, in Section 5.4.3. (For screening techniques, see, for example, Harlow, E. and Lane, eds., 1988, "Antibodies: A Laboratory Manual", Cold Spring Harbor Press, Cold Spring Harbor.) In cases where the mutation results in an expressed gene product with altered function (e.g., as a result of a missense mutation), a polyclonal set of antibodies are likely to cross-react with the mutant gene product. Library clones detected via their reaction with such labeled antibodies can be purified and subjected to sequence analysis as described in this Section, above.

5.4.2. DIFFERENTIALLY EXPRESSED AND PATHWAY GENE PRODUCTS

Differentially expressed and pathway gene products include those proteins encoded by the differentially expressed and pathway gene sequences described in Section 5.4.1, above. Specifically, differentially expressed and pathway gene products may include differentially expressed and pathway gene polypeptides encoded by the differentially expressed and pathway gene sequences contained in the clones listed in Table 2, above, as deposited with the ATCC, or contained in the coding regions of the genes to which DNA sequences disclosed herein (in FIGS. 1-5) or contained in the clones, listed in Table 2, as deposited with the ATCC, belong, for example.

In addition, differentially expressed and pathway gene products may include proteins that represent functionally equivalent gene products. Such an equivalent differentially expressed or pathway gene product may contain deletions, additions or substitutions of amino acid residues within the amino acid sequence encoded by the differentially expressed or pathway gene sequences described, above, in Section 5.4.1, but which result in a silent change, thus producing a functionally equivalent differentially expressed on pathway gene product. Amino acid substitutions may be made on the basis of similarity in polarity, charge,

solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved.

For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, 5 phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic 10 acid and glutamic acid. "Functionally equivalent", as utilized herein, refers to a protein capable of exhibiting a substantially similar in vivo activity as the endogenous differentially expressed or pathway gene products encoded by the differentially expressed or pathway gene sequences 15 described in Section 5.4.1, above. Alternatively, when utilized as part of assays such as those described, below, in Section 5.5, "functionally equivalent" may refer to peptides capable of interacting with other cellular or extracellular molecules in a manner substantially similar to the way in 20 which the corresponding portion of the endogenous differentially expressed or pathway gene product would.

The differentially expressed or pathway gene products may be produced by recombinant DNA technology using techniques well known in the art. Thus, methods for 25 preparing the differentially expressed or pathway gene polypeptides and peptides of the invention by expressing nucleic acid encoding differentially expressed or pathway gene sequences are described herein. Methods which are well known to those skilled in the art can be used to construct 30 expression vectors containing differentially expressed or pathway gene protein coding sequences and appropriate transcriptional/translational control signals. These methods include, for example, in vitro recombinant DNA techniques, synthetic techniques and in vivo recombination/genetic 35 recombination. See, for example, the techniques described in Sambrook et al., 1989, *supra*, and Ausubel et al., 1989, *supra*. Alternatively, RNA capable of encoding differentially

expressed or pathway gene protein sequences may be chemically synthesized using, for example, synthesizers. See, for example, the techniques described in "Oligonucleotide Synthesis", 1984, Gait, M.J. ed., IRL Press, Oxford, which is
5 incorporated by reference herein in its entirety.

A variety of host-expression vector systems may be utilized to express the differentially expressed or pathway gene coding sequences of the invention. Such host-expression systems represent vehicles by which the coding sequences of
10 interest may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, exhibit the differentially expressed or pathway gene protein of the invention in situ. These include but are not limited to
15 microorganisms such as bacteria (e.g., *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing differentially expressed or pathway gene protein coding sequences; yeast (e.g. *Saccharomyces*, *Pichia*) transformed with recombinant
20 yeast expression vectors containing the differentially expressed or pathway gene protein coding sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing the differentially expressed or pathway gene protein coding sequences; plant
25 cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing differentially expressed or pathway gene protein coding sequences; or
30 mammalian cell systems (e.g. COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5K
35 promoter).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use

intended for the differentially expressed or pathway gene protein being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of antibodies or to screen peptide libraries, for example, 5 vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the *E. coli* expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:1791), in which the differentially expressed or pathway 10 gene protein coding sequence may be ligated individually into the vector in frame with the *lac Z* coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic Acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 264:5503-5509); and the like. pGEX 15 vectors may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free 20 glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene protein can be released from the GST moiety.

In a preferred embodiment, full length cDNA 25 sequences are appended with in-frame Bam HI sites at the amino terminus and Eco RI sites at the carboxyl terminus using standard PCR methodologies (Innis et al., 1990, *supra*) and ligated into the pGEX-2TK vector (Pharmacia, Uppsala, Sweden). The resulting cDNA construct contains a kinase 30 recognition site at the amino terminus for radioactive labelling and glutathione S-transferase sequences at the carboxyl terminus for affinity purification (Nilsson, et al., 1985, EMBO J. 4: 1075; Zabeau and Stanley, 1982, EMBO J. 1: 1217.

35 In an insect system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda*

cells. The differentially expressed or pathway gene coding sequence may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of differentially expressed or pathway gene coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect *Spodoptera frugiperda* cells in which the inserted gene is expressed. (E.g., see Smith et al., 1983, J. Virol. 46: 584; Smith, U.S. Patent No. 4,215,051).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the differentially expressed or pathway gene coding sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing differentially expressed or pathway gene protein in infected hosts. (E.g., See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 81:3655-3659). Specific initiation signals may also be required for efficient translation of inserted differentially expressed or pathway gene coding sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire differentially expressed or pathway gene, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of the differentially expressed or pathway gene coding sequence is inserted, exogenous translational control

signals, including, perhaps, the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous
5 translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bittner et al., 1987, Methods in
10 Enzymol. 153:516-544).

In a preferred embodiment, cDNA sequences encoding the full-length open reading frames are ligated into pCMV β replacing the β -galactosidase gene such that cDNA expression is driven by the CMV promoter (Alam, 1990, Anal. Biochem.
15 188: 245-254; MacGregor & Caskey, 1989, Nucl. Acids Res. 17: 2365; Norton & Corrin, 1985, Mol. Cell. Biol. 5: 281).

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific
20 fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins.
25 Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the
30 gene product may be used. Such mammalian host cells include but are not limited to CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, etc.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell
35 lines which stably express the differentially expressed or pathway gene protein may be engineered. Rather than using expression vectors which contain viral origins of

replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker.

5 Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably

10 integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the differentially expressed or pathway gene protein. Such engineered cell lines may be

15 particularly useful in screening and evaluation of compounds that affect the endogenous activity of the differentially expressed or pathway gene protein.

A number of selection systems may be used, including but not limited to the herpes simplex virus

20 thymidine kinase (Wigler, et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell 22:817) genes can be employed in tk⁻, hgp^rt⁻ or ap^rt⁻ cells,

25 respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare, et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid

30 (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1); and hyg^r, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30:147) genes.

35 An alternative fusion protein system allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht, et al., 1991, Proc. Natl.

Acad. Sci. USA 88: 8972-8976). In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the gene's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni^{2+} nitriloacetic acid-agarose columns and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

When used as a component in assay systems such as those described, below, in Section 5.5, the differentially expressed or pathway gene protein may be labeled, either directly or indirectly, to facilitate detection of a complex formed between the differentially expressed or pathway gene protein and a test substance. Any of a variety of suitable labeling systems may be used including but not limited to radioisotopes such as ^{125}I ; enzyme labelling systems that generate a detectable colorimetric signal or light when exposed to substrate; and fluorescent labels.

Where recombinant DNA technology is used to produce the differentially expressed or pathway gene protein for such assay systems, it may be advantageous to engineer fusion proteins that can facilitate labeling, immobilization and/or detection.

Indirect labeling involves the use of a protein, such as a labeled antibody, which specifically binds to either a differentially expressed or pathway gene product. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments and fragments produced by an Fab expression library.

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5.4.3. DIFFERENTIALLY EXPRESSED OR PATHWAY GENE PRODUCT ANTIBODIES

Described herein are methods for the production of antibodies capable of specifically recognizing one or more differentially expressed or pathway gene epitopes. Such antibodies may include, but are not limited to polyclonal

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antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab')₂ fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-
5 binding fragments of any of the above. Such antibodies may be used, for example, in the detection of a fingerprint, target, or pathway gene in a biological sample, or, alternatively, as a method for the inhibition of abnormal target gene activity. Thus, such antibodies may be utilized
10 as part of cardiovascular disease treatment methods, and/or may be used as part of diagnostic techniques whereby patients may be tested for abnormal levels of fingerprint, target, or pathway gene proteins, or for the presence of abnormal forms of the such proteins.

15 For the production of antibodies to a differentially expressed or pathway gene, various host animals may be immunized by injection with a differentially expressed or pathway gene protein, or a portion thereof. Such host animals may include but are not limited to rabbits,
20 mice, and rats, to name but a few. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic
25 polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*.

In a preferred embodiment, peptide sequences
30 corresponding to amino sequences of target gene products were selected and submitted to Research Genetics (Huntsville, AL) for synthesis and antibody production. Peptides were modified as described (Tam, J.P., 1988, Proc. Natl. Acad. Sci. USA 85: 5409-5413; Tam, J.P., and Zavala, F., 1989, J.
35 Immunol. Methods 124: 53-61; Tam, J.P., and Lu, Y.A., 1989, Proc. Natl. Acad. Sci. USA 86: 9084-9088), emulsified in an equal volume of Freund's adjuvant and injected into rabbits

at 3 to 4 subcutaneous dorsal sites for a total volume of 1.0 ml (0.5 mg peptide) per immunization. The animals were boosted after 2 and 6 weeks and bled at weeks 4, 8, and 10. The blood was allowed to clot and serum was collected by centrifugation. The generation of polyclonal antibodies against the fchd545 gene product is described in detail in the example in Section 10, below.

Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen, such as target gene product, or an antigenic functional derivative thereof. For the production of polyclonal antibodies, host animals such as those described above, may be immunized by injection with differentially expressed or pathway gene product supplemented with adjuvants as also described above.

Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, may be obtained by any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to the hybridoma technique of Kohler and Milstein, (1975, Nature 256:495-497; and U.S. Patent No. 4,376,110), the human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today 4:72; Cole et al., 1983, Proc. Natl. Acad. Sci. USA 80:2026-2030), and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies And Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this invention may be cultivated in vitro or in vivo. Production of high titers of mAbs in vivo makes this the presently preferred method of production.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci., 81:6851-6855; Neuberger et al., 1984, Nature, 312:604-608; Takeda et al., 1985, Nature, 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a

human antibody molecule of appropriate biological activity can be used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region.

Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778; Bird, 1988, Science 242:423-426; Huston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; and Ward et al., 1989, Nature 334:544-546) can be adapted to produce differentially expressed or pathway gene-single chain antibodies. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments which recognize specific epitopes may be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')₂ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')₂ fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, Science, 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

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5.4.4. CELL- AND ANIMAL-BASED MODEL SYSTEMS

Described herein are cell- and animal-based systems which act as models for cardiovascular disease. These systems may be used in a variety of applications. For example, the cell- and animal-based model systems may be used to further characterize differentially expressed and pathway genes, as described, above, in Section 5.3. Such further characterization may, for example, indicate that a differentially expressed gene is a target gene. Second, such assays may be utilized as part of screening strategies designed to identify compounds which are capable of ameliorating cardiovascular disease symptoms, as described,

below, in Section 5.5.4. Thus, the animal- and cell-based models may be used to identify drugs, pharmaceuticals, therapies and interventions which may be effective in treating cardiovascular disease. In addition, as described
5 in detail, below, in Section 5.7.1, such animal models may be used to determine the LD₅₀ and the ED₅₀ in animal subjects, and such data can be used to determine the in vivo efficacy of potential cardiovascular disease treatments.

10 5.4.4.1. ANIMAL-BASED SYSTEMS

Animal-based model systems of cardiovascular disease may include, but are not limited to, non-recombinant and engineered transgenic animals.

Non-recombinant animal models for cardiovascular
15 disease may include, for example, genetic models. Such genetic cardiovascular disease models may include, for example, apoB or apoR deficient pigs (Rapacz, et al., 1986, Science 234:1573-1577) and Watanabe heritable hyperlipidemic (WHHL) rabbits (Kita et al., 1987, Proc. Natl. Acad. Sci USA
20 84: 5928-5931).

Non-recombinant, non-genetic animal models of atherosclerosis may include, for example, pig, rabbit, or rat models in which the animal has been exposed to either chemical wounding through dietary supplementation of LDL, or
25 mechanical wounding through balloon catheter angioplasty, for example.

Additionally, animal models exhibiting cardiovascular disease symptoms may be engineered by utilizing, for example, target gene sequences such as those
30 described, above, in Section 5.4.1, in conjunction with techniques for producing transgenic animals that are well known to those of skill in the art. For example, target gene sequences may be introduced into, and overexpressed in, the genome of the animal of interest, or, if endogenous target
35 gene sequences are present, they may either be overexpressed or, alternatively, be disrupted in order to underexpress or inactivate target gene expression, such as described for the

disruption of apoE in mice (Plump et al., 1992, Cell 71: 343-353).

In order to overexpress a target gene sequence, the coding portion of the target gene sequence may be ligated to
5 a regulatory sequence which is capable of driving gene expression in the animal and cell type of interest. Such regulatory regions will be well known to those of skill in the art, and may be utilized in the absence of undue experimentation.

10 For underexpression of an endogenous target gene sequence, such a sequence may be isolated and engineered such that when reintroduced into the genome of the animal of interest, the endogenous target gene alleles will be inactivated. Preferably, the engineered target gene sequence
15 is introduced via gene targeting such that the endogenous target sequence is disrupted upon integration of the engineered target gene sequence into the animal's genome. Gene targeting is discussed, below, in this Section.

Animals of any species, including, but not limited
20 to, mice, rats, rabbits, guinea pigs, pigs, micro-pigs, goats, and non-human primates, e.g., baboons, monkeys, and chimpanzees may be used to generate cardiovascular disease animal models.

Any technique known in the art may be used to
25 introduce a target gene transgene into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to pronuclear microinjection (Hoppe, P.C. and Wagner, T.E., 1989, U.S. Pat. No. 4,873,191); retrovirus mediated gene transfer into germ lines
30 (Van der Putten et al., 1985, Proc. Natl. Acad. Sci., USA 82:6148-6152); gene targeting in embryonic stem cells (Thompson et al., 1989, Cell 56:313-321); electroporation of embryos (Lo, 1983, Mol Cell. Biol. 3:1803-1814); and sperm-mediated gene transfer (Lavitrano et al., 1989, Cell 57:717-
35 723); etc. For a review of such techniques, see Gordon, 1989, Transgenic Animals, Intl. Rev. Cytol. 115:171-229, which is incorporated by reference herein in its entirety.

The present invention provides for transgenic animals that carry the transgene in all their cells, as well as animals which carry the transgene in some, but not all their cells, i.e., mosaic animals. The transgene may be
5 integrated as a single transgene or in concatamers, e.g., head-to-head tandems or head-to-tail tandems. The transgene may also be selectively introduced into and activated in a particular cell type by following, for example, the teaching of Lasko et al. (Lasko, M. et al., 1992, Proc. Natl. Acad.
10 Sci. USA 89: 6232-6236). The regulatory sequences required for such a cell-type specific activation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art. When it is desired that the target gene transgene be integrated into the chromosomal site
15 of the endogenous target gene, gene targeting is preferred. Briefly, when such a technique is to be utilized, vectors containing some nucleotide sequences homologous to the endogenous target gene of interest are designed for the purpose of integrating, via homologous recombination with
20 chromosomal sequences, into and disrupting the function of the nucleotide sequence of the endogenous target gene. The transgene may also be selectively introduced into a particular cell type, thus inactivating the endogenous gene of interest in only that cell type, by following, for
25 example, the teaching of Gu et al. (Gu, et al., 1994, Science 265: 103-106). The regulatory sequences required for such a cell-type specific inactivation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art. Recombinant methods for
30 expressing target genes are described in Section 5.4.2, above.

Once transgenic animals have been generated, the expression of the recombinant target gene and protein may be assayed utilizing standard techniques. Initial screening may
35 be accomplished by Southern blot analysis or PCR techniques to analyze animal tissues to assay whether integration of the transgene has taken place. The level of mRNA expression of

the transgene in the tissues of the transgenic animals may also be assessed using techniques which include but are not limited to Northern blot analysis of tissue samples obtained from the animal, in situ hybridization analysis, and RT-PCR. 5 Samples of target gene-expressing tissue, may also be evaluated immunocytochemically using antibodies specific for the target gene transgene gene product of interest.

The target gene transgenic animals that express target gene mRNA or target gene transgene peptide (detected 10 immunocytochemically, using antibodies directed against the target gene product's epitopes) at easily detectable levels should then be further evaluated to identify those animals which display characteristic cardiovascular disease symptoms. Such symptoms may include, for example, increased prevalence 15 and size of fatty streaks and/or cardiovascular disease plaques.

Additionally, specific cell types within the transgenic animals may be analyzed and assayed for cellular phenotypes characteristic of cardiovascular disease. In the 20 case of monocytes, such phenotypes may include but are not limited to increases in rates of LDL uptake, adhesion to endothelial cells, transmigration, foam cell formation, fatty streak formation, and production of foam cell specific products. Cellular phenotype assays are discussed in detail 25 in Section 5.4.4.2, below. Further, such cellular phenotypes may include a particular cell type's fingerprint pattern of expression as compared to known fingerprint expression profiles of the particular cell type in animals exhibiting cardiovascular disease symptoms. Fingerprint profiles are 30 described in detail in Section 5.8.1, below. Such transgenic animals serve as suitable model systems for cardiovascular disease.

Once target gene transgenic founder animals are produced, they may be bred, inbred, outbred, or crossbred to 35 produce colonies of the particular animal. Examples of such breeding strategies include but are not limited to: outbreeding of founder animals with more than one integration

site in order to establish separate lines; inbreeding of separate lines in order to produce compound target gene transgenics that express the target gene transgene of interest at higher levels because of the effects of additive
5 expression of each target gene transgene; crossing of heterozygous transgenic animals to produce animals homozygous for a given integration site in order both to augment expression and eliminate the possible need for screening of animals by DNA analysis; crossing of separate homozygous
10 lines to produce compound heterozygous or homozygous lines; breeding animals to different inbred genetic backgrounds so as to examine effects of modifying alleles on expression of the target gene transgene and the development of cardiovascular disease symptoms. One such approach is to
15 cross the target gene transgenic founder animals with a wild type strain to produce an F1 generation that exhibits cardiovascular disease symptoms. The F1 generation may then be inbred in order to develop a homozygous line, if it is found that homozygous target gene transgenic animals are
20 viable.

5.4.4.2. CELL-BASED ASSAYS

Cells that contain and express target gene sequences which encode target gene protein, and, further,
25 exhibit cellular phenotypes associated with cardiovascular disease, may be utilized to identify compounds that exhibit anti-cardiovascular disease activity.

Such cells may include non-recombinant monocyte cell lines, such as U937 (ATCC# CRL-1593), THP-1 (ATCC# TIB-
30 202), and P388D1 (ATCC# TIB-63); endothelial cells such as HUVEC's and bovine aortic endothelial cells (BAEC's); as well as generic mammalian cell lines such as HeLa cells and COS cells, e.g., COS-7 (ATCC# CRL-1651). Further, such cells may include recombinant, transgenic cell lines. For example, the
35 cardiovascular disease animal models of the invention, discussed, above, in Section 5.4.4.1, may be used to generate cell lines, containing one or more cell types involved in

cardiovascular disease, that can be used as cell culture models for this disorder. While primary cultures derived from the cardiovascular disease transgenic animals of the invention may be utilized, the generation of continuous cell lines is preferred. For examples of techniques which may be used to derive a continuous cell line from the transgenic animals, see Small et al., 1985, Mol. Cell Biol. 5:642-648.

Alternatively, cells of a cell type known to be involved in cardiovascular disease may be transfected with sequences capable of increasing or decreasing the amount of target gene expression within the cell. For example, target gene sequences may be introduced into, and overexpressed in, the genome of the cell of interest, or, if endogenous target gene sequences are present, they may be either overexpressed or, alternatively disrupted in order to underexpress or inactivate target gene expression.

In order to overexpress a target gene sequence, the coding portion of the target gene sequence may be ligated to a regulatory sequence which is capable of driving gene expression in the cell type of interest. Such regulatory regions will be well known to those of skill in the art, and may be utilized in the absence of undue experimentation. Recombinant methods for expressing target genes are described in Section 5.4.2, above.

For underexpression of an endogenous target gene sequence, such a sequence may be isolated and engineered such that when reintroduced into the genome of the cell type of interest, the endogenous target gene alleles will be inactivated. Preferably, the engineered target gene sequence is introduced via gene targeting such that the endogenous target sequence is disrupted upon integration of the engineered target gene sequence into the cell's genome. Transfection of host cells with target genes is discussed, above, in Section 5.4.4.1.

Cells treated with compounds or transfected with target genes can be examined for phenotypes associated with cardiovascular disease. In the case of monocytes, such

phenotypes include but are not limited to increases in rates of LDL uptake, adhesion to endothelial cells, transmigration, foam cell formation, fatty streak formation, and production by foam cells of growth factors such as bFGF, IGF-I, VEGF, IL-1, M-CSF, TGF β , TGF α , TNF α , HB-EGF, PDGF, IFN- γ , and GM-CSF. Transmigration rates, for example, may be measured using the in vitro system of Navab et al., described in Section 5.1.1.3, above, by quantifying the number of monocytes that migrate across the endothelial monolayer and into the collagen layer of the subendothelial space.

Similarly, HUVEC's can be treated with test compounds or transfected with genetically engineered target genes described in Section 5.4.2, above. The HUVEC's can then be examined for phenotypes associated with cardiovascular disease, including, but not limited to changes in cellular morphology, cell proliferation, cell migration, and mononuclear cell adhesion; or for the effects on production of other proteins involved in cardiovascular disease such as ICAM, VCAM, PDGF- β , and E-selectin.

Transfection of target gene sequence nucleic acid may be accomplished by utilizing standard techniques. See, for example, Ausubel, 1989, *supra*. Transfected cells should be evaluated for the presence of the recombinant target gene sequences, for expression and accumulation of target gene mRNA, and for the presence of recombinant target gene protein production. In instances wherein a decrease in target gene expression is desired, standard techniques may be used to demonstrate whether a decrease in endogenous target gene expression and/or in target gene product production is achieved.

5.5. SCREENING ASSAYS FOR COMPOUNDS THAT INTERACT WITH THE TARGET GENE PRODUCT AND/OR MODULATE TARGET GENE EXPRESSION

The following assays are designed to identify compounds that bind to target gene products, bind to other cellular or extracellular proteins that interact with a

target gene product, and interfere with the interaction of the target gene product with other cellular or extracellular proteins. Such compounds can act as the basis for amelioration of such cardiovascular diseases as
5 atherosclerosis, ischemia/reperfusion, hypertension, restenosis, and arterial inflammation by modulating the activity of the protein products of target genes. Such compounds may include, but are not limited to peptides, antibodies, or small organic or inorganic compounds. Methods
10 for the identification of such compounds are described in Section 5.5.1, below. Such compounds may also include other cellular proteins. Methods for the identification of such cellular proteins are described, below, in Section 5.5.2.

Compounds identified via assays such as those
15 described herein may be useful, for example, in elaborating the biological function of the target gene product, and for ameliorating cardiovascular disease. In instances whereby a cardiovascular disease condition results from an overall lower level of target gene expression and/or target gene
20 product in a cell or tissue, compounds that interact with the target gene product may include compounds which accentuate or amplify the activity of the bound target gene protein. Such compounds would bring about an effective increase in the level of target gene product activity, thus ameliorating
25 symptoms.

In some cases, a target gene observed to be up-regulated under disease conditions may be exerting a protective effect. Compounds that enhance the expression of such up-regulated genes, or the activity of their gene
30 products, would also ameliorate disease symptoms, especially in individuals whose target gene is not normally up-regulated.

In other instances mutations within the target gene may cause aberrant types or excessive amounts of target gene
35 proteins to be made which have a deleterious effect that leads to cardiovascular disease. Similarly, physiological conditions may cause an excessive increase in target gene

expression leading to cardiovascular disease. In such cases, compounds that bind target gene protein may be identified that inhibit the activity of the bound target gene protein. Assays for testing the effectiveness of compounds, identified
5 by, for example, techniques such as those described in this Section are discussed, below, in Section 5.5.4.

5.5.1. IN VITRO SCREENING ASSAYS FOR COMPOUNDS
THAT BIND TO THE TARGET GENE PRODUCT

10 In vitro systems may be designed to identify compounds capable of binding the target gene of the invention. Such compounds may include, but are not limited to, peptides made of D-and/or L-configuration amino acids (in, for example, the form of random peptide libraries; see
15 e.g., Lam, K.S. et al., 1991, Nature 354:82-84), phosphopeptides (in, for example, the form of random or partially degenerate, directed phosphopeptide libraries; see, e.g., Songyang, Z. et al., 1993, Cell 72:767-778), antibodies, and small organic or inorganic molecules.
20 Compounds identified may be useful, for example, in modulating the activity of target gene proteins, preferably mutant target gene proteins, may be useful in elaborating the biological function of the target gene protein, may be utilized in screens for identifying compounds that disrupt
25 normal target gene interactions, or may in themselves disrupt such interactions. For instance, the example in Section 12, below, describes the interaction between the rchd534 protein and the fchd540 protein. Compounds that disrupt the interaction between these two proteins may be useful in the
30 treatment of cardiovascular disease.

The principle of the assays used to identify compounds that bind to the target gene protein involves preparing a reaction mixture of the target gene protein and the test compound under conditions and for a time sufficient
35 to allow the two components to interact and bind, thus forming a complex which can be removed and/or detected in the reaction mixture. These assays can be conducted in a variety

of ways. For example, one method to conduct such an assay would involve anchoring the target gene or the test substance onto a solid phase and detecting target gene/test substance complexes anchored on the solid phase at the end of the
5 reaction. In one embodiment of such a method, the target gene protein may be anchored onto a solid surface, and the test compound, which is not anchored, may be labeled, either directly or indirectly.

In practice, microtitre plates are conveniently
10 utilized. The anchored component may be immobilized by non-covalent or covalent attachments. Non-covalent attachment may be accomplished simply by coating the solid surface with a solution of the protein and drying. Alternatively, an immobilized antibody, preferably a monoclonal antibody,
15 specific for the protein may be used to anchor the protein to the solid surface. The surfaces may be prepared in advance and stored.

In order to conduct the assay, the nonimmobilized component is added to the coated surface containing the
20 anchored component. After the reaction is complete, unreacted components are removed (e.g., by washing) under conditions such that any complexes formed will remain immobilized on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number
25 of ways. Where the previously nonimmobilized component is pre-labeled, the detection of label immobilized on the surface indicates that complexes were formed. Where the previously nonimmobilized component is not pre-labeled, an indirect label can be used to detect complexes anchored on
30 the surface; e.g., using a labeled antibody specific for the previously nonimmobilized component (the antibody, in turn, may be directly labeled or indirectly labeled with a labeled anti-Ig antibody).

Alternatively, a reaction can be conducted in a
35 liquid phase, the reaction products separated from unreacted components, and complexes detected; e.g., using an immobilized antibody specific for target gene product or the

test compound to anchor any complexes formed in solution, and a labeled antibody specific for the other component of the possible complex to detect anchored complexes.

Compounds that are shown to bind to a particular target gene product through one of the methods described above can be further tested for their ability to elicit a biochemical response from the target gene protein. A particular embodiment is described herein for receptor proteins involved in signal transduction. Compounds that interact with a target gene product receptor domain, can be screened for their ability to function as ligands, i.e., to bind to the receptor protein in a manner that triggers the signal transduction pathway. Useful receptor fragments or analogs in the invention are those which interact with ligand. The receptor component can be assayed functionally, i.e., for its ability to bind ligand and mobilize Ca^{2+} (see below). These assays include, as components, ligand and a recombinant target gene product (or a suitable fragment or analog) configured to permit detection of binding.

For example, and not by way of limitation, a recombinant receptor may be used to screen for ligands by its ability to mediate ligand-dependent mobilization of calcium. Cells, preferably myeloma cells or *Xenopus* oocytes, transfected with a target gene expression vector (constructed according to the methods described in Section 5.4.2, above) are loaded with FURA-2 or INDO-1 by standard techniques. Mobilization of Ca^{2+} induced by ligand is measured by fluorescence spectroscopy as previously described (Grynkiewicz et al., 1985, *J. Biol. Chem.* 260:3440). Ligands that react with the target gene product receptor domain, therefore, can be identified by their ability to produce a fluorescent signal. Their receptor binding activities can be quantified and compared by measuring the level of fluorescence produced over background. Identification of ligand, and measuring the activity of the ligand-receptor complex, leads to the identification of antagonists of this interaction, as described in Section 5.5.3, below. Such

antagonists are useful in the treatment of cardiovascular disease.

5 5.5.2. ASSAYS FOR CELLULAR OR EXTRACELLULAR
 PROTEINS THAT INTERACT WITH THE TARGET
 GENE PRODUCT

Any method suitable for detecting protein-protein interactions may be employed for identifying novel target protein-cellular or extracellular protein interactions. 10 These methods are outlined in Section 5.2., supra, for the identification of pathway genes, and may be utilized herein with respect to the identification of proteins which interact with identified target proteins. In such a case, the target gene serves as the known "bait" gene.

15 The example presented in Section 12, below, demonstrates the use of this method to detect the interaction between the rchd534 protein and the fchd540 protein, which both had been identified as target proteins.

20 5.5.3. ASSAYS FOR COMPOUNDS THAT INTERFERE WITH
 INTERACTION BETWEEN TARGET GENE PRODUCT
 AND OTHER COMPOUNDS

The target gene proteins of the invention may, in vivo, interact with one or more cellular or extracellular proteins. Such proteins may include, but are not limited to, 25 those proteins identified via methods such as those described, above, in Section 5.5.2. For the purposes of this discussion, target gene products and such cellular and extracellular proteins are referred to herein as "binding partners". Compounds that disrupt such interactions may be 30 useful in regulating the activity of the target gene proteins, especially mutant target gene proteins. Such compounds may include, but are not limited to molecules such as antibodies, peptides, and the like described in Section 5.5.1. above.

35 The basic principle of the assay systems used to identify compounds that interfere with the interaction between the target gene protein, and its cellular or

extracellular protein binding partner or partners involves preparing a reaction mixture containing the target gene protein and the binding partner under conditions and for a time sufficient to allow the two proteins to interact and
5 bind, thus forming a complex. In order to test a compound for inhibitory activity, the reaction mixture is prepared in the presence and absence of the test compound. The test compound may be initially included in the reaction mixture or may be added at a time subsequent to the addition of target
10 gene and its cellular or extracellular binding partner. Control reaction mixtures are incubated without the test compound or with a placebo. The formation of any complexes between the target gene protein and the cellular or extracellular binding partner is then detected. The
15 formation of a complex in the control reaction, but not in the reaction mixture containing the test compound, indicates that the compound interferes with the interaction of the target gene protein and the interactive binding partner protein. Additionally, complex formation within reaction
20 mixtures containing the test compound and a normal target gene protein may also be compared to complex formation within reaction mixtures containing the test compound and mutant target gene protein. This comparison may be important in those cases wherein it is desirable to identify compounds
25 that disrupt interactions of mutant but not normal target gene proteins.

The assay for compounds that interfere with the interaction of the binding partners can be conducted in a heterogeneous or homogeneous format. Heterogeneous assays
30 involve anchoring one of the binding partners onto a solid phase and detecting complexes anchored on the solid phase at the end of the reaction. In homogeneous assays, the entire reaction is carried out in a liquid phase. In either approach, the order of addition of reactants can be varied to
35 obtain different information about the compounds being tested. For example, test compounds that interfere with the interaction between the binding partners, e.g., by

competition, can be identified by conducting the reaction in the presence of the test substance; i.e., by adding the test substance to the reaction mixture prior to or simultaneously with the target gene protein and interactive cellular or extracellular protein. Alternatively, test compounds that disrupt preformed complexes, e.g. compounds with higher binding constants that displace one of the binding partners from the complex, can be tested by adding the test compound to the reaction mixture after complexes have been formed.

10 The various formats are described briefly below.

In a heterogeneous assay system, either the target gene protein or the interactive cellular or extracellular binding partner protein, is anchored onto a solid surface, and its binding partner, which is not anchored, is labeled, either directly or indirectly. In practice, microtitre plates are conveniently utilized. The anchored species may be immobilized by non-covalent or covalent attachments. Non-covalent attachment may be accomplished simply by coating the solid surface with a solution of the protein and drying.

20 Alternatively, an immobilized antibody specific for the protein may be used to anchor the protein to the solid surface. The surfaces may be prepared in advance and stored.

In order to conduct the assay, the binding partner of the immobilized species is exposed to the coated surface with or without the test compound. After the reaction is complete, unreacted components are removed (e.g., by washing) and any complexes formed will remain immobilized on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the binding partner was pre-labeled, the detection of label immobilized on the surface indicates that complexes were formed. Where the binding partner is not pre-labeled, an indirect label can be used to detect complexes anchored on the surface; e.g., using a labeled antibody specific for the binding partner (the antibody, in turn, may be directly labeled or indirectly labeled with a labeled anti-Ig antibody). Depending upon the order of addition of reaction

components, test compounds which inhibit complex formation or which disrupt preformed complexes can be detected.

Alternatively, the reaction can be conducted in a liquid phase in the presence or absence of the test compound, the reaction products separated from unreacted components, and complexes detected; e.g., using an immobilized antibody specific for one binding partner to anchor any complexes formed in solution, and a labeled antibody specific for the other binding partner to detect anchored complexes. Again, depending upon the order of addition of reactants to the liquid phase, test compounds which inhibit complex or which disrupt preformed complexes can be identified.

In an alternate embodiment of the invention, a homogeneous assay can be used. In this approach, a preformed complex of the target gene protein and the interactive cellular or extracellular protein is prepared in which one of the binding partners is labeled, but the signal generated by the label is quenched due to complex formation (see, e.g., U.S. Patent No. 4,109,496 by Rubenstein which utilizes this approach for immunoassays). The addition of a test substance that competes with and displaces one of the binding partners from the preformed complex will result in the generation of a signal above background. In this way, test substances which disrupt target gene protein-cellular or extracellular protein interaction can be identified.

In a particular embodiment, the target gene protein can be prepared for immobilization using recombinant DNA techniques described in Section 5.4.2, *supra*. For example, the target gene coding region can be fused to a glutathione-S-transferase (GST) gene, using a fusion vector such as pGEX-5X-1, in such a manner that its binding activity is maintained in the resulting fusion protein. The interactive cellular or extracellular protein can be purified and used to raise a monoclonal antibody, using methods routinely practiced in the art and described above, in Section 5.4.3. This antibody can be labeled with the radioactive isotope ¹²⁵I, for example, by methods routinely practiced in the art.

In a heterogeneous assay, e.g., the GST-target gene fusion protein can be anchored to glutathione-agarose beads. The interactive cellular or extracellular binding partner protein can then be added in the presence or absence of the test
5 compound in a manner that allows interaction and binding to occur. At the end of the reaction period, unbound material can be washed away, and the labeled monoclonal antibody can be added to the system and allowed to bind to the complexed binding partners. The interaction between the target gene
10 protein and the interactive cellular or extracellular binding partner protein can be detected by measuring the amount of radioactivity that remains associated with the glutathione-agarose beads. A successful inhibition of the interaction by the test compound will result in a decrease in measured
15 radioactivity.

Alternatively, the GST-target gene fusion protein and the interactive cellular or extracellular binding partner protein can be mixed together in liquid in the absence of the solid glutathione-agarose beads. The test compound can be
20 added either during or after the binding partners are allowed to interact. This mixture can then be added to the glutathione-agarose beads and unbound material is washed away. Again the extent of inhibition of the binding partner interaction can be detected by adding the labeled antibody
25 and measuring the radioactivity associated with the beads.

In another embodiment of the invention, these same techniques can be employed using peptide fragments that correspond to the binding domains of the target gene protein and the interactive cellular or extracellular protein,
30 respectively, in place of one or both of the full length proteins. Any number of methods routinely practiced in the art can be used to identify and isolate the protein's binding site. These methods include, but are not limited to, mutagenesis of one of the genes encoding the proteins and
35 screening for disruption of binding in a co-immunoprecipitation assay. Compensating mutations in the target gene can be selected. Sequence analysis of the genes

encoding the respective proteins will reveal the mutations that correspond to the region of the protein involved in interactive binding. Alternatively, one protein can be anchored to a solid surface using methods described in this
5 Section above, and allowed to interact with and bind to its labeled binding partner, which has been treated with a proteolytic enzyme, such as trypsin. After washing, a short, labeled peptide comprising the binding domain may remain associated with the solid material, which can be isolated and
10 identified by amino acid sequencing. Also, once the gene coding for the for the cellular or extracellular protein is obtained, short gene segments can be engineered to express peptide fragments of the protein, which can then be tested for binding activity and purified or synthesized.

15 For example, and not by way of limitation, target gene can be anchored to a solid material as described above in this Section by making a GST-target gene fusion protein and allowing it to bind to glutathione agarose beads. The interactive cellular or extracellular binding partner protein
20 can be labeled with a radioactive isotope, such as ³⁵S, and cleaved with a proteolytic enzyme such as trypsin. Cleavage products can then be added to the anchored GST-target gene fusion protein and allowed to bind. After washing away unbound peptides, labeled bound material, representing the
25 cellular or extracellular binding partner protein binding domain, can be eluted, purified, and analyzed for amino acid sequence by techniques well known in the art; e.g., using the Edman degradation procedure (see e.g., Creighton, 1983, Proteins: Structures and Molecular Principles, W.H. Freeman &
30 Co., N.Y., pp. 34-49). Peptides so identified can be produced, using techniques well known in the art, either synthetically (see e.g., Creighton, 1983, *supra* at pp. 50-60) or, if the gene has already been isolated, by using recombinant DNA technology, as described in Section 5.4.2,
35 *supra*.

A particular embodiment of the invention features a method of screening candidate compounds for their ability to antagonize the interaction between ligand and the receptor domain of a target gene product. The method involves: a) 5 mixing a candidate antagonist compound with a first compound which includes a recombinant target gene product comprising a receptor domain (or ligand-binding fragment or analog) on the one hand and with a second compound which includes ligand on the other hand; b) determining whether the first and second 10 compounds bind; and c) identifying antagonistic compounds as those which interfere with the binding of the first compound to the second compound and/or which reduce the ligand-mediated release of intracellular Ca^{++} .

By an "antagonist" is meant a molecule which 15 inhibits a particular activity, in this case, the ability of ligand to interact with a target gene product receptor domain and/or to trigger the biological events resulting from such an interaction (e.g., release of intracellular Ca^{++}).

Preferred therapeutics include antagonists, e.g., peptide 20 fragments (particularly, fragments derived from the N-terminal extracellular domain), antibodies (particularly, antibodies which recognize and bind the N-terminal extracellular domain), or drugs, which block ligand or target gene product function by interfering with the ligand-receptor 25 interaction.

Because the receptor component of the target gene product can be produced by recombinant techniques and because candidate antagonists may be screened in vitro, the instant invention provides a simple and rapid approach to the 30 identification of useful therapeutics.

Specific receptor fragments of interest include any portions of the target gene products that are capable of interaction with ligand, for example, all or part of the N-terminal extracellular domain. Such portions include the 35 transmembrane segments and portions of the receptor deduced to be extracellular. Such fragments may be useful as antagonists (as described above), and are also useful as

immunogens for producing antibodies which neutralize the activity of the target gene product in vivo (e.g., by interfering with the interaction between the receptor and ligand; see below). Extracellular regions may be identified
5 by comparison with related proteins of similar structure, useful regions are those exhibiting homology to the extracellular domains of well-characterized members of the family.

Alternatively, from the primary amino acid
10 sequence, the secondary protein structure and, therefore, the extracellular domain regions may be deduced semi-empirically using a hydrophobicity/hydrophilicity calculation such as the Chou-Fasman method (see, e.g., Chou and Fasman, *Ann. Rev. Biochem.* 47:251, 1978). Hydrophilic domains, particularly
15 ones surrounded by hydrophobic stretches (e.g., transmembrane domains) present themselves as strong candidates for extracellular domains. Finally, extracellular domains may be identified experimentally using standard enzymatic digest analysis, e.g., tryptic digest analysis.

20 Candidate fragments (e.g., all or part of the transmembrane segments or any extracellular fragment) are tested for interaction with ligand by the assays described herein (e.g., the assay described above). Such fragments are also tested for their ability to antagonize the interaction
25 between ligand and its endogenous receptor using the assays described herein. Analogs of useful receptor fragments (as described above) may also be produced and tested for efficacy as screening components or antagonists (using the assays described herein); such analogs are also considered to be
30 useful in the invention.

Of particular interest are receptor fragments encompassing the extracellular main-terminal domain (or a ligand binding fragment thereof). Also of interest are the target gene product extracellular loops. Peptide fragments
35 derived from these extracellular loops may also be used as antagonists, particularly if the loops cooperate with the amino-terminal domain to facilitate ligand binding.

Alternatively, such loops and extracellular N-terminal domain (as well as the full length target gene product) provide immunogens for producing anti-target gene product antibodies.

Binding of ligand to its receptor may be assayed by
5 any of the methods described above in Section 5.5.1.

Preferably, cells expressing recombinant target gene product (or a suitable target gene product fragment or analog) are immobilized on a solid substrate (e.g., the wall of a microtitre plate or a column) and reacted with detectably-
10 labelled ligand (as described above). Binding is assayed by the detection label in association with the receptor component (and, therefore, in association with the solid substrate). Binding of labelled ligand to receptor-bearing cells is used as a "control" against which antagonist assays
15 are measured. The antagonist assays involve incubation of the target gene product-bearing cells with an appropriate amount of candidate antagonist. To this mix, an equivalent amount to labelled ligand is added. An antagonist useful in the invention specifically interferes with labelled ligand
20 binding to the immobilized receptor-expressing cells.

An antagonist is then tested for its ability to interfere with ligand function, i.e., to specifically interfere with labelled ligand binding without resulting in signal transduction normally mediated by the receptor. To
25 test this using a functional assay, stably transfected cell lines containing the target gene product can be produced as described herein and reporter compounds such as the calcium binding agent, FURA-2, loaded into the cytoplasm by standard techniques. Stimulation of the heterologous target gene
30 product with ligand or another agonist leads to intracellular calcium release and the concomitant fluorescence of the calcium-FURA-2 complex. This provides a convenient means for measuring agonist activity. Inclusion of potential
antagonists along with ligand allows for the screening and
35 identification of authentic receptor antagonists as those which effectively block ligand binding without producing fluorescence (i.e., without causing the mobilization of

intracellular Ca^{++}). Such an antagonist may be expected to be a useful therapeutic agent for cardiovascular disorders.

Appropriate candidate antagonists include target gene product fragments, particularly fragments containing a
5 ligand-binding portion adjacent to or including one or more transmembrane segments or an extracellular domain of the receptor (described above); such fragments would preferably including five or more amino acids. Other candidate antagonists include analogs of ligand and other peptides as
10 well as non-peptide compounds and anti-target gene product antibodies designed or derived from analysis of the receptor.

5.5.4. ASSAYS FOR AMELIORATION OF CARDIOVASCULAR DISEASE SYMPTOMS

15 Any of the binding compounds, including but not limited to compounds such as those identified in the foregoing assay systems, may be tested for the ability to ameliorate cardiovascular disease symptoms. Cell-based and animal model-based assays for the identification of compounds
20 exhibiting such an ability to ameliorate cardiovascular disease symptoms are described below.

First, cell-based systems such as those described, above, in Section 5.4.4.2., may be used to identify compounds which may act to ameliorate cardiovascular disease symptoms.
25 For example, such cell systems may be exposed to a compound, suspected of exhibiting an ability to ameliorate cardiovascular disease symptoms, at a sufficient concentration and for a time sufficient to elicit such an amelioration of cardiovascular disease symptoms in the
30 exposed cells. After exposure, the cells are examined to determine whether one or more of the cardiovascular disease cellular phenotypes has been altered to resemble a more normal or more wild type, non-cardiovascular disease phenotype. For example, and not by way of limitation, in the
35 case of monocytes, such more normal phenotypes may include but are not limited to decreased rates of LDL uptake, adhesion to endothelial cells, transmigration, foam cell

formation, fatty streak formation, and production by foam cells of growth factors such as bFGF, IGF-I, VEGF, IL-1, M-CSF, TGF β , TGF α , TNF α , HB-EGF, PDGF, IFN- γ , and GM-CSF.

Transmigration rates, for example, may be measured using the
5 in vitro system of Navab et al., described in Section 5.1.1.3, above, by quantifying the number of monocytes that migrate across the endothelial monolayer and into the collagen layer of the subendothelial space.

In addition, animal-based cardiovascular disease
10 systems, such as those described, above, in Section 5.4.4.1, may be used to identify compounds capable of ameliorating cardiovascular disease symptoms. Such animal models may be used as test substrates for the identification of drugs, pharmaceuticals, therapies, and interventions which may be
15 effective in treating cardiovascular disease. For example, animal models may be exposed to a compound, suspected of exhibiting an ability to ameliorate cardiovascular disease symptoms, at a sufficient concentration and for a time sufficient to elicit such an amelioration of cardiovascular
20 disease symptoms in the exposed animals. The response of the animals to the exposure may be monitored by assessing the reversal of disorders associated with cardiovascular disease, for example, by counting the number of atherosclerotic plaques and/or measuring their size before and after
25 treatment.

With regard to intervention, any treatments which reverse any aspect of cardiovascular disease symptoms should be considered as candidates for human cardiovascular disease therapeutic intervention. Dosages of test agents may be
30 determined by deriving dose-response curves, as discussed in Section 5.7.1, below.

Additionally, gene expression patterns may be utilized to assess the ability of a compound to ameliorate cardiovascular disease symptoms. For example, the expression
35 pattern of one or more fingerprint genes may form part of a "fingerprint profile" which may be then be used in such an assessment. "Fingerprint profile", as used herein, refers to

the pattern of mRNA expression obtained for a given tissue or cell type under a given set of conditions. Such conditions may include, but are not limited to, atherosclerosis, ischemia/reperfusion, hypertension, restenosis, and arterial inflammation, including any of the control or experimental conditions described in the paradigms of Section 5.1.1, above. Fingerprint profiles may be generated, for example, by utilizing a differential display procedure, as discussed, above, in Section 5.1.2, Northern analysis and/or RT-PCR.

10 Any of the gene sequences described, above, in Section 5.4.1. may be used as probes and/or PCR primers for the generation and corroboration of such fingerprint profiles.

Fingerprint profiles may be characterized for known states, either cardiovascular disease or normal, within the cell- and/or animal-based model systems. Subsequently, these known fingerprint profiles may be compared to ascertain the effect a test compound has to modify such fingerprint profiles, and to cause the profile to more closely resemble that of a more desirable fingerprint.

20 For example, administration of a compound may cause the fingerprint profile of a cardiovascular disease model system to more closely resemble the control system. Administration of a compound may, alternatively, cause the fingerprint profile of a control system to begin to mimic a cardiovascular disease state. Such a compound may, for example, be used in further characterizing the compound of interest, or may be used in the generation of additional animal models.

30 5.5.5. MONITORING OF EFFECTS DURING CLINICAL TRIALS

Monitoring the influence of compounds on cardiovascular disease states may be applied not only in basic drug screening, but also in clinical trials. In such clinical trials, the expression of a panel of genes that have been discovered in one of the paradigms described in Section

5.1.1.1 through 5.1.1.6 may be used as a "read out" of a particular drug's effect on a cardiovascular disease state.

For example, and not by way of limitation, Paradigm A provides for the identification of fingerprint genes that are up-regulated in monocytes treated with oxidized LDL. Thus, to study the effect of anti-oxidant drugs, for example, in a clinical trial, blood may be drawn from patients before and at different stages during treatment with such a drug. Their monocytes may then be isolated and RNA prepared and analyzed by differential display as described in Sections 6.1.1 and 6.1.2. The levels of expression of these fingerprint genes may be quantified by Northern blot analysis or RT-PCR, as described in Section 6.1.2, or by one of the methods described in Section 5.8.1, or alternatively by measuring the amount of protein produced, by one of the methods described in Section 5.8.2. In this way, the fingerprint profiles may serve as surrogate markers indicative of the physiological response of monocytes that have taken up oxidized LDL. Accordingly, this response state may be determined before, and at various points during, drug treatment. This method is described in further detail in the example in Section 8, below. Specifically, the up-regulation of fchd602 and fchd605 under treatment with oxidized LDL provides a fingerprint profile for monocytes under oxidative stress. The fchd602 and fchd605 genes can serve, therefore, as surrogate markers during clinical treatment of cardiovascular disease. Accordingly, the influence of anti-oxidant drugs on oxidative potential is measured by recording the differential display of fchd602 and fchd605 in the monocytes of patients undergoing clinical treatment.

5.5.6. ASSAYS FOR COMPOUNDS THAT MODULATE EXPRESSION OF TARGET GENES

Compounds and other substances that modulate expression of target genes can be screened using in vitro cellular systems. In a manner analogous to the monitoring of

compounds clinical samples described in Section 5.5.5, above, a sample of cells, such as a tissue culture is exposed to a test substance. Appropriate tissue culture cells include, but are not limited to, human umbilical vein endothelial cells (HUVECs), bovine aortic endothelial cells (BAECs), and 293 cells (embryonic human kidney cells). The RNA is then extracted from the cells. The level of transcription of a specific target gene can be detected using, for example, standard RT-PCR amplification techniques and/or Northern analysis (as described in the example in Section 6.1.2, below). Alternatively, the level of target protein production can be assayed by using antibodies that detect the target gene protein, as described in Section 5.5.1, above. The level of expression is compared to a control cell sample which was not exposed to the test substance.

Compounds that can be screened for modulation of expression of the target gene include, but are not limited to, small inorganic or organic molecules, peptides, such as peptide hormones analogs, steroid hormones, analogs of such hormones, and other proteins. Compounds that down-regulate expression include, but are not limited to, oligonucleotides that are complementary to the 5'-end of the mRNA of the target gene and inhibit transcription by forming triple helix structures, and ribozymes or antisense molecules which inhibit translation of the target gene mRNA. Techniques and strategies for designing such down-regulating test compounds are described in detail in Section 5.6, below.

5.6. COMPOUNDS AND METHODS FOR TREATMENT OF CARDIOVASCULAR DISEASE

Described below are methods and compositions whereby cardiovascular disease symptoms may be ameliorated. Certain cardiovascular diseases are brought about, at least in part, by an excessive level of gene product, or by the presence of a gene product exhibiting an abnormal or excessive activity. As such, the reduction in the level and/or activity of such gene products would bring about the

amelioration of cardiovascular disease symptoms. Techniques for the reduction of target gene expression levels or target gene product activity levels are discussed in Section 5.6.1, below.

5 Alternatively, certain other cardiovascular diseases are brought about, at least in part, by the absence or reduction of the level of gene expression, or a reduction in the level of a gene product's activity. As such, an increase in the level of gene expression and/or the activity
10 of such gene products would bring about the amelioration of cardiovascular disease symptoms.

In some cases, the up-regulation of a gene in a disease state reflects a protective role for that gene product in responding to the disease condition. Enhancement
15 of such a target gene's expression, or the activity of the target gene product, will reinforce the protective effect it exerts. Some cardiovascular disease states may result from an abnormally low level of activity of such a protective gene. In these cases also, an increase in the level of gene
20 expression and/or the activity of such gene products would bring about the amelioration of cardiovascular disease symptoms. Techniques for increasing target gene expression levels or target gene product activity levels are discussed in Section 5.6.2, below.

25

5.6.1. COMPOUNDS THAT INHIBIT EXPRESSION,
SYNTHESIS OR ACTIVITY OF MUTANT TARGET
GENE ACTIVITY

As discussed above, target genes involved in
30 cardiovascular disease disorders can cause such disorders via an increased level of target gene activity. As summarized in Table 1, above, and detailed in the examples in Sections 6 and 7, below, a number of genes have been demonstrated to be up-regulated in monocytes and endothelial cells under disease
35 conditions. Specifically, fchd602 and fchd605 are each up-regulated in monocytes treated with oxidized LDL. Furthermore, fchd540 is up-regulated in endothelial cells

subjected to shear stress. In some cases, such up-regulation may have a causative or exacerbating effect on the disease state. A variety of techniques may be utilized to inhibit the expression, synthesis, or activity of such target genes
5 and/or proteins.

For example, compounds such as those identified through assays described, above, in Section 5.5, which exhibit inhibitory activity, may be used in accordance with the invention to ameliorate cardiovascular disease symptoms.
10 As discussed in Section 5.5, above, such molecules may include, but are not limited to small organic molecules, peptides, antibodies, and the like. Inhibitory antibody techniques are described, below, in Section 5.6.1.2.

For example, compounds can be administered that
15 compete with endogenous ligand for a transmembrane target gene product. The resulting reduction in the amount of ligand-bound target gene transmembrane protein will modulate cell physiology. Compounds that can be particularly useful for this purpose include, for example, soluble proteins or
20 peptides, such as peptides comprising one or more of the extracellular domains, or portions and/or analogs thereof, of the target gene product, including, for example, soluble fusion proteins such as Ig-tailed fusion proteins. (For a discussion of the production of Ig-tailed fusion proteins,
25 see, for example, U.S. Patent No. 5,116,964.). Alternatively, compounds, such as ligand analogs or antibodies, that bind to the target gene product receptor site, but do not activate the protein, (e.g., receptor-ligand antagonists) can be effective in inhibiting target gene
30 product activity.

Further, antisense and ribozyme molecules which inhibit expression of the target gene may also be used in accordance with the invention to inhibit the aberrant target gene activity. Such techniques are described, below, in
35 Section 5.6.1.1. Still further, also as described, below, in Section 5.6.1.1, triple helix molecules may be utilized in inhibiting the aberrant target gene activity.

5.6.1.1. INHIBITORY ANTISENSE, RIBOZYME, TRIPLE HELIX, AND GENE INACTIVATION APPROACHES

Among the compounds which may exhibit the ability to ameliorate cardiovascular disease symptoms are antisense, ribozyme, and triple helix molecules. Such molecules may be designed to reduce or inhibit mutant target gene activity. Techniques for the production and use of such molecules are well known to those of skill in the art.

Antisense RNA and DNA molecules act to directly block the translation of mRNA by hybridizing to targeted mRNA and preventing protein translation.

Antisense approaches involve the design of oligonucleotides (either DNA or RNA) that are complementary to target gene mRNA. The antisense oligonucleotides will bind to the complementary target gene mRNA transcripts and prevent translation. Absolute complementarity, although preferred, is not required. A sequence "complementary" to a portion of an RNA, as referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex; in the case of double-stranded antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with an RNA it may contain and still form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

Oligonucleotides that are complementary to the 5' end of the message, e.g., the 5' untranslated sequence up to and including the AUG initiation codon, should work most efficiently at inhibiting translation. However, sequences complementary to the 3' untranslated sequences of mRNAs have recently shown to be effective at inhibiting translation of

mRNAs as well. See generally, Wagner, R., 1994, Nature 372:333-335. Thus, oligonucleotides complementary to either the 5'- or 3'- non-translated, non-coding regions of the target gene could be used in an antisense approach to inhibit translation of endogenous target gene mRNA. Oligonucleotides complementary to the 5' untranslated region of the mRNA should include the complement of the AUG start codon. Antisense oligonucleotides complementary to mRNA coding regions are less efficient inhibitors of translation but could be used in accordance with the invention. Whether designed to hybridize to the 5'-, 3'- or coding region of target gene mRNA, antisense nucleic acids should be at least six nucleotides in length, and are preferably oligonucleotides ranging from 6 to about 50 nucleotides in length. In specific aspects the oligonucleotide is at least 10 nucleotides, at least 17 nucleotides, at least 25 nucleotides or at least 50 nucleotides.

Regardless of the choice of target sequence, it is preferred that *in vitro* studies are first performed to quantitate the ability of the antisense oligonucleotide to inhibit gene expression. It is preferred that these studies utilize controls that distinguish between antisense gene inhibition and nonspecific biological effects of oligonucleotides. It is also preferred that these studies compare levels of the target RNA or protein with that of an internal control RNA or protein. Additionally, it is envisioned that results obtained using the antisense oligonucleotide are compared with those obtained using a control oligonucleotide. It is preferred that the control oligonucleotide is of approximately the same length as the test oligonucleotide and that the nucleotide sequence of the oligonucleotide differs from the antisense sequence no more than is necessary to prevent specific hybridization to the target sequence.

The oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be

modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule, hybridization, etc. The oligonucleotide may include other appended groups such as peptides (e.g., for targeting host cell receptors in vivo), or agents facilitating transport across the cell membrane (see, e.g., Letsinger et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:6553-6556; Lemaitre et al., 1987, Proc. Natl. Acad. Sci. 84:648-652; PCT Publication No. WO88/09810, published December 15, 1988) or the blood-brain barrier (see, e.g., PCT Publication No. WO89/10134, published April 25, 1988), hybridization-triggered cleavage agents. (See, e.g., Krol et al., 1988, BioTechniques 6:958-976) or intercalating agents. (See, e.g., Zon, 1988, Pharm. Res. 5:539-549). To this end, the oligonucleotide may be conjugated to another molecule, e.g., a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

The antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl) uracil, (acp3)w, and 2,6-diaminopurine.

The antisense oligonucleotide may also comprise at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose.

5 In yet another embodiment, the antisense oligonucleotide comprises at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an
10 alkyl phosphotriester, and a formacetal or analog thereof.

In yet another embodiment, the antisense oligonucleotide is an α -anomeric oligonucleotide. An α -anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β -units,
15 the strands run parallel to each other (Gautier et al., 1987, Nucl. Acids Res. 15:6625-6641). The oligonucleotide is a 2'-O-methylribonucleotide (Inoue et al., 1987, Nucl. Acids Res. 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue et al., 1987, FEBS Lett. 215:327-330).

20 Oligonucleotides of the invention may be synthesized by standard methods known in the art, e.g. by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be synthesized by the
25 method of Stein et al. (1988, Nucl. Acids Res. 16:3209), methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:7448-7451), etc.

While antisense nucleotides complementary to the target
30 gene coding region sequence could be used, those complementary to the transcribed untranslated region are most preferred.

Specific antisense oligonucleotides for the rchd534 gene and fchd540 gene are described in the Example in Section 13,
35 below.

The antisense molecules should be delivered to cells which express the target gene *in vivo*, e.g., endothelial

cells. A number of methods have been developed for delivering antisense DNA or RNA to cells; e.g., antisense molecules can be injected directly into the tissue site, or modified antisense molecules, designed to target the desired
5 cells (e.g., antisense linked to peptides or antibodies that specifically bind receptors or antigens expressed on the target cell surface) can be administered systemically.

However, it is often difficult to achieve intracellular concentrations of the antisense sufficient to suppress
10 translation of endogenous mRNAs. Therefore a preferred approach utilizes a recombinant DNA construct in which the antisense oligonucleotide is placed under the control of a strong pol III or pol II promoter. The use of such a construct to transfect target cells in the patient will
15 result in the transcription of sufficient amounts of single stranded RNAs that will form complementary base pairs with the endogenous target gene transcripts and thereby prevent translation of the target gene mRNA. For example, a vector can be introduced in vivo such that it is taken up by a cell
20 and directs the transcription of an antisense RNA. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art.
25 Vectors can be plasmid, viral, or others known in the art, used for replication and expression in mammalian cells. Expression of the sequence encoding the antisense RNA can be by any promoter known in the art to act in mammalian, preferably human cells. Such promoters can be inducible or
30 constitutive. Such promoters include but are not limited to: the SV40 early promoter region (Beruoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter
35 (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42), etc. Any type of

plasmid, cosmid, YAC or viral vector can be used to prepare the recombinant DNA construct which can be introduced directly into the tissue site; e.g., atherosclerotic vascular tissue. Alternatively, viral vectors can be used which
5 selectively infect the desired tissue, in which case administration may be accomplished by another route (e.g., systemically).

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of
10 ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by an endonucleolytic cleavage. Ribozyme molecules designed to catalytically cleave target gene mRNA transcripts can also be used to prevent translation of target gene mRNA and
15 expression of target gene. (See, e.g., PCT International Publication WO90/11364, published October 4, 1990; Sarver et al., 1990, Science 247:1222-1225). While ribozymes that cleave mRNA at site specific recognition sequences can be used to destroy target gene mRNAs, the use of hammerhead
20 ribozymes is preferred. Hammerhead ribozymes cleave mRNAs at locations dictated by flanking regions that form complementary base pairs with the target mRNA. The sole requirement is that the target mRNA have the following sequence of two bases: 5'-UG-3'. The construction and
25 production of hammerhead ribozymes is well known in the art and is described more fully in Haseloff and Gerlach, 1988, Nature, 334:585-591. For example, there are hundreds of potential hammerhead ribozyme cleavage sites within the nucleotide sequence of rchd534 and fchd540 cDNA. Preferably
30 the ribozyme is engineered so that the cleavage recognition site is located near the 5' end of the target mRNA; i.e., to increase efficiency and minimize the intracellular accumulation of non-functional mRNA transcripts.

Specific hammerhead ribozymes molecules for the rchd534
35 and fchd540 genes are described in the Example in Section 13, below.

The ribozymes of the present invention also include RNA endoribonucleases (hereinafter "Cech-type ribozymes") such as the one which occurs naturally in *Tetrahymena Thermophila* (known as the IVS, or L-19 IVS RNA) and which has been
5 extensively described by Thomas Cech and collaborators (Zaug, et al., 1984, *Science*, 224:574-578; Zaug and Cech, 1986, *Science*, 231:470-475; Zaug, et al., 1986, *Nature*, 324:429-433; published International patent application No. WO 88/04300 by University Patents Inc.; Been and Cech, 1986,
10 *Cell*, 47:207-216). The Cech-type ribozymes have an eight base pair active site which hybridizes to a target RNA sequence whereafter cleavage of the target RNA takes place. The invention encompasses those Cech-type ribozymes which target eight base-pair active site sequences that are present
15 in target gene.

As in the antisense approach, the ribozymes can be composed of modified oligonucleotides (e.g. for improved stability, targeting, etc.) and should be delivered to cells which express the target gene *in vivo*, e.g., endothelial
20 cells. A preferred method of delivery involves using a DNA construct "encoding" the ribozyme under the control of a strong constitutive pol III or pol II promoter, so that transfected cells will produce sufficient quantities of the ribozyme to destroy endogenous target gene messages and
25 inhibit translation. Because ribozymes, unlike antisense molecules, are catalytic, a lower intracellular concentration is required for efficiency.

Nucleic acid molecules to be used in triple helix formation for the inhibition of transcription should be
30 single stranded and composed of deoxyribonucleotides. The base composition of these oligonucleotides must be designed to promote triple helix formation via Hoogsteen base pairing rules, which generally require sizeable stretches of either purines or pyrimidines to be present on one strand of a
35 duplex. Nucleotide sequences may be pyrimidine-based, which will result in TAT and CGC* triplets across the three associated strands of the resulting triple helix. The

pyrimidine-rich molecules provide base complementarity to a purine-rich region of a single strand of the duplex in a parallel orientation to that strand. In addition, nucleic acid molecules may be chosen that are purine-rich, for example, containing a stretch of G residues. These molecules will form a triple helix with a DNA duplex that is rich in GC pairs, in which the majority of the purine residues are located on a single strand of the targeted duplex, resulting in GGC triplets across the three strands in the triplex.

10 Alternatively, the potential sequences that can be targeted for triple helix formation may be increased by creating a so called "switchback" nucleic acid molecule. Switchback molecules are synthesized in an alternating 5'-3', 3'-5' manner, such that they base pair with first one strand
15 of a duplex and then the other, eliminating the necessity for a sizeable stretch of either purines or pyrimidines to be present on one strand of a duplex.

It is possible that the antisense, ribozyme, and/or triple helix molecules described herein may reduce or inhibit
20 the transcription (triple helix) and/or translation (antisense, ribozyme) of mRNA produced by both normal and mutant target gene alleles. In order to ensure that substantially normal levels of target gene activity are maintained, nucleic acid molecules that encode and express
25 target gene polypeptides exhibiting normal activity may be introduced into cells via gene therapy methods such as those described, below, in Section 5.7. that do not contain sequences susceptible to whatever antisense, ribozyme, or triple helix treatments are being utilized. Alternatively,
30 it may be preferable to coadminister normal target gene protein into the cell or tissue in order to maintain the requisite level of cellular or tissue target gene activity.

Endogenous target gene expression can also be reduced by inactivating or "knocking out" the target gene or its
35 promoter using targeted homologous recombination. (E.g., see Smithies et al., 1985, Nature 317:230-234; Thomas & Capecchi, 1987, Cell 51:503-512; Thompson et al., 1989 Cell 5:313-321;

each of which is incorporated by reference herein in its entirety). For example, a mutant, non-functional target (or a completely unrelated DNA sequence) flanked by DNA homologous to the endogenous target gene (either the coding regions or regulatory regions of the target gene) can be used, with or without a selectable marker and/or a negative selectable marker, to transfect cells that express target *in vivo*. Insertion of the DNA construct, via targeted homologous recombination, results in inactivation of the target gene. Such approaches can be adapted for use in humans provided the recombinant DNA constructs are directly administered or targeted to the required site *in vivo* using appropriate viral vectors, e.g., vectors for delivery vascular tissue.

Alternatively, endogenous target gene expression can be reduced by targeting deoxyribonucleotide sequences complementary to the regulatory region of the target gene (i.e., the target promoter and/or enhancers) to form triple helical structures that prevent transcription of the target gene in target cells in the body. (See generally, Helene, C. 1991, Anticancer Drug Des., 6(6):569-84; Helene, C., et al., 1992, Ann, N.Y. Acad. Sci., 660:27-36; and Maher, L.J., 1992, Bioassays 14(12):807-15).

In yet another embodiment of the invention, the activity of a target can be reduced using a "dominant negative" approach to effectuate reduction in cardiovascular disease symptoms. For example, if two gene products interact, such as the rchd534 and fchd540 proteins, then the presence of a mutant version of one or both of these proteins in the cell can reduce the overall pool of complexes consisting of entirely wild-type proteins. In this manner, the overall level of activity resulting from the rchd534/fchd540 protein interaction can be reduced.

5.6.1.2. ANTIBODIES FOR TARGET GENE PRODUCTS

Antibodies that are both specific for target gene protein and interfere with its activity may be used to

inhibit target gene function. Such antibodies may be generated using standard techniques described in Section 5.4.3., *supra*, against the proteins themselves or against peptides corresponding to portions of the proteins. Such
5 antibodies include but are not limited to polyclonal, monoclonal, Fab fragments, single chain antibodies, chimeric antibodies, etc.

In instances where the target gene protein is intracellular and whole antibodies are used, internalizing
10 antibodies may be preferred. However, lipofectin liposomes may be used to deliver the antibody or a fragment of the Fab region which binds to the target gene epitope into cells. Where fragments of the antibody are used, the smallest inhibitory fragment which binds to the target protein's
15 binding domain is preferred. For example, peptides having an amino acid sequence corresponding to the domain of the variable region of the antibody that binds to the target gene protein may be used. Such peptides may be synthesized chemically or produced via recombinant DNA technology using
20 methods well known in the art (*e.g.*, see Creighton, 1983, *supra*; and Sambrook et al., 1989, *supra*). Alternatively, single chain neutralizing antibodies which bind to intracellular target gene epitopes may also be administered. Such single chain antibodies may be administered, for
25 example, by expressing nucleotide sequences encoding single-chain antibodies within the target cell population by utilizing, for example, techniques such as those described in Marasco et al. (Marasco, W. et al., 1993, Proc. Natl. Acad. Sci. USA 90:7889-7893).

30 In some instances, the target gene protein is extracellular, or is a transmembrane protein, such as the fchd545 and fchd602 gene products. Antibodies that are specific for one or more extracellular domains of these gene products, for example, and that interfere with its activity,
35 are particularly useful in treating cardiovascular disease. Such antibodies are especially efficient because they can access the target domains directly from the bloodstream.

Any of the administration techniques described, below in Section 5.7 which are appropriate for peptide administration may be utilized to effectively administer inhibitory target gene antibodies to their site of action.

5

5.6.2. METHODS FOR RESTORING OR ENHANCING
TARGET GENE ACTIVITY

Target genes that cause cardiovascular disease may be underexpressed within cardiovascular disease situations.

10 As summarized in Table 1, above, and detailed in the example in Section 7, below, several genes are now known to be down-regulated in endothelial cells under disease conditions. Specifically, fchd531 and fchd545 are down-regulated in endothelial cells subjected to shear stress. Alternatively,
15 the activity of target gene products may be decreased, leading to the development of cardiovascular disease symptoms. Such down-regulation of target gene expression or decrease of target gene product activity might have a causative or exacerbating effect on the disease state.

20 In some cases, target genes that are up-regulated in the disease state might be exerting a protective effect. As summarized in Table 1, above, and detailed in the examples in Sections 6 and 7, below, a number of genes are now known to be up-regulated in monocytes and endothelial cells under
25 disease conditions. Specifically, fchd602 and fchd605 are each up-regulated in monocytes treated with oxidized LDL. Furthermore, fchd540 is up-regulated in endothelial cells subjected to shear stress. A variety of techniques may be utilized to increase the expression, synthesis, or activity
30 of such target genes and/or proteins, for those genes that exert a protective effect in response to disease conditions.

Described in this Section are methods whereby the level of target gene activity may be increased to levels wherein cardiovascular disease symptoms are ameliorated. The
35 level of gene activity may be increased, for example, by either increasing the level of target gene product present or

by increasing the level of active target gene product which is present.

For example, a target gene protein, at a level sufficient to ameliorate cardiovascular disease symptoms may be administered to a patient exhibiting such symptoms. Any of the techniques discussed, below, in Section 5.7, may be utilized for such administration. One of skill in the art will readily know how to determine the concentration of effective, non-toxic doses of the normal target gene protein, utilizing techniques such as those described, below, in Section 5.7.1.

Additionally, RNA sequences encoding target gene protein may be directly administered to a patient exhibiting cardiovascular disease symptoms, at a concentration sufficient to produce a level of target gene protein such that cardiovascular disease symptoms are ameliorated. Any of the techniques discussed, below, in Section 5.7, which achieve intracellular administration of compounds, such as, for example, liposome administration, may be utilized for the administration of such RNA molecules. The RNA molecules may be produced, for example, by recombinant techniques such as those described, above, in Section 5.4.2.

Further, patients may be treated by gene replacement therapy. One or more copies of a normal target gene, or a portion of the gene that directs the production of a normal target gene protein with target gene function, may be inserted into cells using vectors which include, but are not limited to adenovirus, adeno-associated virus, and retrovirus vectors, in addition to other particles that introduce DNA into cells, such as liposomes. Additionally, techniques such as those described above may be utilized for the introduction of normal target gene sequences into human cells.

Cells, preferably, autologous cells, containing normal target gene expressing gene sequences may then be introduced or reintroduced into the patient at positions which allow for the amelioration of cardiovascular disease

symptoms. Such cell replacement techniques may be preferred, for example, when the target gene product is a secreted, extracellular gene product.

5 5.7. PHARMACEUTICAL PREPARATIONS AND METHODS OF ADMINISTRATION

 The identified compounds that inhibit target gene expression, synthesis and/or activity can be administered to a patient at therapeutically effective doses to treat or
10 ameliorate cardiovascular disease. A therapeutically effective dose refers to that amount of the compound sufficient to result in amelioration of symptoms of cardiovascular disease.

15 5.7.1. EFFECTIVE DOSE

 Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD₅₀ (the dose lethal to 50% of the population) and the
20 ED₅₀ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD₅₀/ED₅₀. Compounds which exhibit large therapeutic indices are preferred. While compounds that exhibit toxic
25 side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

 The data obtained from the cell culture assays and
30 animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED₅₀ with little or no toxicity. The dosage may vary within this range depending upon the dosage form
35 employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially

from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

10 5.7.2. FORMULATIONS AND USE

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers or excipients.

15 Thus, the compounds and their physiologically acceptable salts and solvates may be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral or rectal administration.

20 For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinised maize starch, polyvinylpyrrolidone or
25 hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulphate).
30 The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid
35 preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g., sorbitol syrup, cellulose derivatives or

hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (e.g., methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

Preparations for oral administration may be suitably formulated to give controlled release of the active compound.

For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g. gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

5 In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for
10 example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

15 The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by
20 instructions for administration.

5.8. DIAGNOSIS OF CARDIOVASCULAR DISEASE ABNORMALITIES

A variety of methods may be employed, utilizing reagents such as fingerprint gene nucleotide sequences
25 described in Section 5.4.1, and antibodies directed against differentially expressed and pathway gene peptides, as described, above, in Sections 5.4.2. (peptides) and 5.4.3. (antibodies). Specifically, such reagents may be used, for example, for the detection of the presence of target gene
30 mutations, or the detection of either over or under expression of target gene mRNA.

The methods described herein may be performed, for example, by utilizing pre-packaged diagnostic kits comprising at least one specific fingerprint gene nucleic acid or anti-
35 fingerprint gene antibody reagent described herein, which may be conveniently used, e.g., in clinical settings, to diagnose

patients exhibiting cardiovascular disease symptoms or at risk for developing cardiovascular disease.

Any cell type or tissue, preferably monocytes, endothelial cells, or smooth muscle cells, in which the fingerprint gene is expressed may be utilized in the diagnostics described below.

5.8.1. DETECTION OF FINGERPRINT GENE NUCLEIC ACIDS

10 DNA or RNA from the cell type or tissue to be analyzed may easily be isolated using procedures which are well known to those in the art. Diagnostic procedures may also be performed "in situ" directly upon tissue sections (fixed and/or frozen) of patient tissue obtained from
15 biopsies or resections, such that no nucleic acid purification is necessary. Nucleic acid reagents such as those described in Section 5.1. may be used as probes and/or primers for such in situ procedures (see, for example, Nuovo, G.J., 1992, PCR in situ hybridization: protocols and
20 applications, Raven Press, NY).

Fingerprint gene nucleotide sequences, either RNA or DNA, may, for example, be used in hybridization or amplification assays of biological samples to detect cardiovascular disease-related gene structures and
25 expression. Such assays may include, but are not limited to, Southern or Northern analyses, single stranded conformational polymorphism analyses, in situ hybridization assays, and polymerase chain reaction analyses. Such analyses may reveal both quantitative aspects of the expression pattern of the
30 fingerprint gene, and qualitative aspects of the fingerprint gene expression and/or gene composition. That is, such aspects may include, for example, point mutations, insertions, deletions, chromosomal rearrangements, and/or activation or inactivation of gene expression.

35 Preferred diagnostic methods for the detection of fingerprint gene-specific nucleic acid molecules may involve for example, contacting and incubating nucleic acids, derived

from the cell type or tissue being analyzed, with one or more labeled nucleic acid reagents as are described in Section 5.1, under conditions favorable for the specific annealing of these reagents to their complementary sequences within the nucleic acid molecule of interest. Preferably, the lengths of these nucleic acid reagents are at least 9 to 30 nucleotides. After incubation, all non-annealed nucleic acids are removed from the nucleic acid:fingerprint molecule hybrid. The presence of nucleic acids from the fingerprint tissue which have hybridized, if any such molecules exist, is then detected. Using such a detection scheme, the nucleic acid from the tissue or cell type of interest may be immobilized, for example, to a solid support such as a membrane, or a plastic surface such as that on a microtitre plate or polystyrene beads. In this case, after incubation, non-annealed, labeled fingerprint nucleic acid reagents of the type described in Section 5.1. are easily removed. Detection of the remaining, annealed, labeled nucleic acid reagents is accomplished using standard techniques well-known to those in the art.

Alternative diagnostic methods for the detection of fingerprint gene specific nucleic acid molecules may involve their amplification, e.g., by PCR (the experimental embodiment set forth in Mullis, K.B., 1987, U.S. Patent No. 4,683,202), ligase chain reaction (Barany, F., 1991, Proc. Natl. Acad. Sci. USA 88:189-193), self sustained sequence replication (Guatelli, J.C. et al., 1990, Proc. Natl. Acad. Sci. USA 87:1874-1878), transcriptional amplification system (Kwoh, D.Y et al., 1989, Proc. Natl. Acad. Sci. USA 86:1173-1177), Q-Beta Replicase (Lizardi, P.M. et al., 1988, Bio/Technology 6:1197), or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well known to those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers.

In one embodiment of such a detection scheme, a cDNA molecule is obtained from an RNA molecule of interest (e.g., by reverse transcription of the RNA molecule into cDNA). Cell types or tissues from which such RNA may be isolated include any tissue in which wild type fingerprint gene is known to be expressed, including, but not limited, to monocytes, endothelium, and/or smooth muscle. A fingerprint sequence within the cDNA is then used as the template for a nucleic acid amplification reaction, such as a PCR amplification reaction, or the like. The nucleic acid reagents used as synthesis initiation reagents (e.g., primers) in the reverse transcription and nucleic acid amplification steps of this method are chosen from among the fingerprint gene nucleic acid reagents described in Section 5.1. The preferred lengths of such nucleic acid reagents are at least 15-30 nucleotides. For detection of the amplified product, the nucleic acid amplification may be performed using radioactively or non-radioactively labeled nucleotides. Alternatively, enough amplified product may be made such that the product may be visualized by standard ethidium bromide staining or by utilizing any other suitable nucleic acid staining method.

In addition to methods which focus primarily on the detection of one nucleic acid sequence, fingerprint profiles, as discussed in Section 5.5.4, may also be assessed in such detection schemes. Fingerprint profiles may be generated, for example, by utilizing a differential display procedure, as discussed, above, in Section 5.1.2, Northern analysis and/or RT-PCR. Any of the gene sequences described, above, in Section 5.4.1. may be used as probes and/or PCR primers for the generation and corroboration of such fingerprint profiles.

5.8.2. DETECTION OF FINGERPRINT GENE PEPTIDES

Antibodies directed against wild type or mutant fingerprint gene peptides, which are discussed, above, in Section 5.4.3, may also be used as cardiovascular disease
5 diagnostics and prognostics, as described, for example, herein. Such diagnostic methods, may be used to detect abnormalities in the level of fingerprint gene protein expression, or abnormalities in the structure and/or tissue, cellular, or subcellular location of fingerprint gene
10 protein. Structural differences may include, for example, differences in the size, electronegativity, or antigenicity of the mutant fingerprint gene protein relative to the normal fingerprint gene protein.

Protein from the tissue or cell type to be analyzed
15 may easily be detected or isolated using techniques which are well known to those of skill in the art, including but not limited to western blot analysis. For a detailed explanation of methods for carrying out western blot analysis, see Sambrook et al, 1989, supra, at Chapter 18. The protein
20 detection and isolation methods employed herein may also be such as those described in Harlow and Lane, for example, (Harlow, E. and Lane, D., 1988, "Antibodies: A Laboratory Manual", Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York), which is incorporated herein by reference
25 in its entirety.

Preferred diagnostic methods for the detection of wild type or mutant fingerprint gene peptide molecules may involve, for example, immunoassays wherein fingerprint gene peptides are detected by their interaction with an anti-
30 fingerprint gene specific peptide antibody.

For example, antibodies, or fragments of antibodies, such as those described, above, in Section 5.4.3, useful in the present invention may be used to quantitatively or qualitatively detect the presence of wild type or mutant
35 fingerprint gene peptides. This can be accomplished, for example, by immunofluorescence techniques employing a fluorescently labeled antibody (see below) coupled with light

microscopic, flow cytometric, or fluorimetric detection. Such techniques are especially preferred if the fingerprint gene peptides are expressed on the cell surface.

The antibodies (or fragments thereof) useful in the present invention may, additionally, be employed histologically, as in immunofluorescence or immunoelectron microscopy, for in situ detection of fingerprint gene peptides. In situ detection may be accomplished by removing a histological specimen from a patient, and applying thereto a labeled antibody of the present invention. The antibody (or fragment) is preferably applied by overlaying the labeled antibody (or fragment) onto a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of the fingerprint gene peptides, but also their distribution in the examined tissue. Using the present invention, those of ordinary skill will readily perceive that any of a wide variety of histological methods (such as staining procedures) can be modified in order to achieve such in situ detection.

Immunoassays for wild type or mutant fingerprint gene peptides typically comprise incubating a biological sample, such as a biological fluid, a tissue extract, freshly harvested cells, or cells which have been incubated in tissue culture, in the presence of a detectably labeled antibody capable of identifying fingerprint gene peptides, and detecting the bound antibody by any of a number of techniques well known in the art.

The biological sample may be brought in contact with and immobilized onto a solid phase support or carrier such as nitrocellulose, or other solid support which is capable of immobilizing cells, cell particles or soluble proteins. The support may then be washed with suitable buffers followed by treatment with the detectably labeled fingerprint gene specific antibody. The solid phase support may then be washed with the buffer a second time to remove unbound antibody. The amount of bound label on solid support may then be detected by conventional means.

By "solid phase support or carrier" is intended any support capable of binding an antigen or an antibody. Well-known supports or carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, 5 natural and modified celluloses, polyacrylamides, gabbros, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the coupled 10 molecule is capable of binding to an antigen or antibody. Thus, the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc. 15 Preferred supports include polystyrene beads. Those skilled in the art will know many other suitable carriers for binding antibody or antigen, or will be able to ascertain the same by use of routine experimentation.

The binding activity of a given lot of anti-wild 20 type or mutant fingerprint gene peptide antibody may be determined according to well known methods. Those skilled in the art will be able to determine operative and optimal assay conditions for each determination by employing routine experimentation.

25 One of the ways in which the fingerprint gene peptide-specific antibody can be detectably labeled is by linking the same to an enzyme and use in an enzyme immunoassay (EIA) (Voller, "The Enzyme Linked Immunosorbent Assay (ELISA)", *Diagnostic Horizons* 2:1-7, 1978, 30 Microbiological Associates Quarterly Publication, Walkersville, MD; Voller, et al., *J. Clin. Pathol.* 31:507-520 (1978); Butler, *Meth. Enzymol.* 73:482-523 (1981); Maggio, (ed.) *Enzyme Immunoassay*, CRC Press, Boca Raton, FL, 1980; Ishikawa, et al., (eds.) *Enzyme Immunoassay*, Kigaku Shoin, 35 Tokyo, 1981). The enzyme which is bound to the antibody will react with an appropriate substrate, preferably a chromogenic substrate, in such a manner as to produce a chemical moiety

which can be detected, for example, by spectrophotometric, fluorimetric or by visual means. Enzymes which can be used to detectably label the antibody include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate, dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase. The detection can be accomplished by colorimetric methods which employ a chromogenic substrate for the enzyme. Detection may also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

Detection may also be accomplished using any of a variety of other immunoassays. For example, by radioactively labeling the antibodies or antibody fragments, it is possible to detect fingerprint gene wild type or mutant peptides through the use of a radioimmunoassay (RIA) (see, for example, Weintraub, B., *Principles of Radioimmunoassays*, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March, 1986, which is incorporated by reference herein). The radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography.

It is also possible to label the antibody with a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wave length, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

The antibody can also be detectably labeled using fluorescence emitting metals such as ^{152}Eu , or others of the lanthanide series. These metals can be attached to the antibody using such metal chelating groups as

diethylenetriaminepentacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

The antibody also can be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound may be used to label the antibody of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in, which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

20 5.8.3. IMAGING CARDIOVASCULAR DISEASE CONDITIONS

In some cases, differentially expressed gene products identified herein may be up-regulated under cardiovascular disease conditions and expressed on the surface of the affected tissue. Such target gene products allow for the non-invasive imaging of damaged or diseased cardiovascular tissue for the purposed of diagnosis and directing of treatment of the disease. For example, such differentially expressed gene products may include but are not limited to atherosclerosis specific adhesion molecules responsible for atherogenesis, or monocyte scavenger receptors that are up-regulated in response to oxidized LDL, which are discussed in Section 2, above. Alternatively, other such surface proteins may be specifically up-regulated in tissues suffering from ischemia/reperfusion or other tissues with atherosclerotic or restenotic lesions.

As described in the example in Section 6, below, fchd602 is a gene that is up-regulated in monocytes under

disease conditions. Furthermore, the fchd602 gene encodes a novel protein containing multiple transmembrane domains. Not only is the fchd602 gene expressed in monocytes, which play a role in the initiation and progression of atherosclerotic lesions, it is also upregulated in monocytes under such disease conditions. The fchd602 gene product, therefore, provides an excellent tool for imaging cardiovascular disease conditions.

This method can be applied in a similar manner to other transmembrane target gene products, such as the fchd545 gene product. As described in the example in Section 7, below, the fchd545 gene encodes a novel anion channel, containing multiple transmembrane domains. Because the fchd545 gene product might be more readily detected in normal tissue, as opposed to tissue in the disease state, it also provides an excellent tool for imaging cardiovascular disease conditions.

An example illustrating the use of this method in accordance with the invention is provided in Section 9, below. Monoclonal and polyclonal antibodies, as described in Section 5.6.1.2, above, which specifically bind to such surface proteins, such as the fchd602 and fchd545 gene products, can be used for the diagnosis of cardiovascular disease by in vivo tissue imaging techniques. Such antibodies raised against the fchd545 gene product are described in detail in the example in Section 10, below. An antibody specific for a target gene product, or preferably an antigen binding fragment thereof, is conjugated to a label (e.g., a gamma emitting radioisotope) which generates a detectable signal and administered to a subject (human or animal) suspected of having cardiovascular disease. After sufficient time to allow the detectably-labeled antibody to localize at the diseased or damaged tissue site (or sites), the signal generated by the label is detected by a photoscanning device. The detected signal is then converted to an image of the tissue. This image makes it possible to

localize the tissue in vivo. This data can then be used to develop an appropriate therapeutic strategy.

Antibody fragments, rather than whole antibody molecules, are generally preferred for use in tissue imaging. 5 Antibody fragments accumulate at the tissue(s) more rapidly because they are distributed more readily than are entire antibody molecules. Thus an image can be obtained in less time than is possible using whole antibody. These fragments are also cleared more rapidly from tissues, resulting in a 10 lower background signal. See, e.g., Haber et al., U.S. Patent No. 4,036,945; Goldenberg et al., U.S. Patent No. 4,331,647. The divalent antigen binding fragment (Fab')₂ and the monovalent Fab are especially preferred. Such fragments can be prepared by digestion of the whole immunoglobulin 15 molecule with the enzymes pepsin or papain according to any of several well known protocols. The types of labels that are suitable for conjugation to a monoclonal antibody for diseased or damaged tissue localization include, but are not limited to radiolabels (i.e., radioisotopes), fluorescent 20 labels and biotin labels.

Among the radioisotopes that can be used to label antibodies or antibody fragments, gamma-emitters, positron-emitters, X-ray-emitters and fluorescence-emitters are suitable for localization. Suitable radioisotopes for 25 labeling antibodies include Iodine-131, Iodine-123, Iodine-125, Iodine-126, Iodine-133, Bromine-77, Indium-111, Indium-113m, Gallium-67, Gallium-68, Ruthenium-95, Ruthenium-97, Ruthenium-103, Ruthenium-105, Mercury-107, Mercury-203, Rhenium-99m, Rhenium-105, Rhenium-101, Tellurium-121m, 30 Tellurium-122m, Tellurium-125m, Thulium-165, Thulium-167, Thulium-168, Technetium-99m and Fluorine-18. The halogens can be used more or less interchangeably as labels since halogen-labeled antibodies and/or normal immunoglobulins would have substantially the same kinetics and distribution 35 and similar metabolism.

The gamma-emitters Indium-111 and Technetium-99m are preferred because these radiometals are detectable with a

gamma camera and have favorable half lives for imaging in vivo. Antibody can be labelled with Indium-111 or Technetium-99m via a conjugated metal chelator, such as DTPA (diethylenetriaminepentaacetic acid). See Krejcarek et al., 5 1977, Biochem. Biophys. Res. Comm. 77:581; Khaw et al., 1980, Science 209:295; Gansow et al., U.S. Patent No. 4,472,509; Hnatowich, U.S. Patent No. 4,479,930, the teachings of which are incorporated herein by reference.

Fluorescent compounds that are suitable for 10 conjugation to a monoclonal antibody include fluorescein sodium, fluorescein isothiocyanate, and Texas Red sulfonyl chloride. See, DeBelder & Wik, 1975, Carbohydrate Research 44:254-257. Those skilled in the art will know, or will be able to ascertain with no more than routine experimentation, 15 other fluorescent compounds that are suitable for labeling monoclonal antibodies.

6. EXAMPLE: IDENTIFICATION OF GENES DIFFERENTIALLY EXPRESSED IN RESPONSE TO PARADIGM A: IN VITRO FOAM CELL PARADIGM

20 According to the invention, differential display may be used to detect genes that are differentially expressed in monocytes that were treated so as to simulate the conditions under which foam cells develop during 25 atherogenesis. By use of Paradigm A, the novel genes fchd602 and fchd605 were identified. Both fchd602 and fchd605 are up-regulated under the disease condition of treatment with oxidized LDL.

The fchd602 gene product contains multiple 30 transmembrane domains, and has sequence similarity to the rat Cl-6 gene, which is induced in regenerating rat liver, is insulin inducible, and also contains multiple transmembrane domains (Diamond, R.H., et al., 1993, J. Biol. Chem. 268: 15185-15192). The fchd605 gene product has sequence 35 similarity to the mouse gly96 gene (Charles, C.H., et al., 1993, Oncogene 8: 797-801), and to EST T49532.

The discovery of the up-regulation of these two genes provides a fingerprint profile, e.g., markers, for monocytes in the process of foam cell formation. This profile can be used in the treatment and diagnosis of cardiovascular disease, including but not limited to atherosclerosis, ischemia/reperfusion, hypertension, restenosis, and arterial inflammation.

Furthermore, as a transmembrane protein, the fchd602 gene product can be readily accessed or detected on the monocyte cell surface by other compounds. It provides, therefore, an excellent target for detection of cardiovascular disease states in diagnostic systems, as well as in the monitoring of the efficacy of compounds in clinical trials. Furthermore, the extracellular domains of this gene product provide targets which allow for the design of especially efficient screening systems for identifying compounds that bind to them. Such compounds can be useful in treating cardiovascular disease by modulating the activity of the transmembrane gene product.

20

6.1. MATERIALS AND METHODS

6.1.1. CELL ISOLATION AND CULTURING

Blood (~200 ml) was drawn into chilled 20 ml vacutainer tubes to which 3 ml of citrate phosphate dextrose (Sigma) was added. Blood was then pooled into 50 ml tubes and spun in the Beckman GS-6R at 1250 RPM for 15 minutes at 4°C. The upper clear layer (~25 ml) was then removed with a pipette and discarded and replaced with the same volume of 4°C PBS. The blood was then mixed, and spun again at 2680 RPM for 15 minutes at 4°C. The upper layer was then removed and discarded, and the buffy coat at the interface was removed in ~5 ml and placed in a separate 50 ml tube, and the pipette was washed with 20 ml PBS. Cells were added to a T flask and stored at 4°C for 16 hours. A small aliquot of the cells were then removed and counted using a hemacytometer. The final red blood cell concentration in the buffy coat population was then adjusted to 1.5×10^9 /ml with PBS, the

cells were added to Leucoprep tubes (Becton Dickinson) after being allowed to come to room temperature, and spun at 2300 RPM for 25 minutes at 25°C. The upper clear layer was removed and discarded and the turbid layer over the gel was removed and pooled in 50 ml tubes. Samples were then diluted to 50 ml with PBS (25°C) and spun at 1000 RPM for 10 minutes. The supernatant was then removed, and the pellet was resuspended in 50 ml PBS. This procedure was repeated 3 more times. After the last spin, the cells were resuspended in a small volume of PBS and counted.

Tissue culture dishes were coated with bovine collagen before monocytes were plated out. 1/6 volume of 7X RPMI (JRH Biosciences) was added to Vitrogen 100 collagen (Celtrix) which was then diluted 1:10 with RPMI to a final concentration of 0.35 mg/ml. Collagen mixture was then added to plates (2.5 ml/100 mm dish) and placed at 37°C for at least one hour to allow for gel formation. After gel formation has taken place, the RPMI was removed and cells were added in RPMI/10% plasma derived serum (PDS). PDS was prepared by drawing blood into chilled evacuated tubes containing 1/10th volume 3.8% sodium citrate. Blood was then transferred into new Sorvall tubes and spun at 14,000-16,000 RPM for 20 minutes at 4°C. Plasma layer was removed and pooled in new tubes to which 1/50th volume 1M CaCl₂ was added. Plasma was mixed and aliquoted into new Sorvall tubes and incubated at 37°C for 2 hours to allow for fibrin clot formation. The clot was then disturbed with a pipette to allow it to contract and tubes were spun at 14,500 RPM for 20 minutes at 25°C. Supernatant was collected, pooled, and heat inactivated at 56°C prior to sterile filtration and freezing.

Purified human monocytes were cultured in 10% PDS/RPMI containing 5 units/ml of Genzyme recombinant human MCSF for 5 days before being treated with LDL, oxidized LDL, acetylated LDL (all LDL at 50 µg/ml), lysophosphatidylcholine (Sigma, 37.5 µM), or homocysteine (Sigma, 1mM). After incubation with these reagents for periods ranging from 2 hours up to 3 days, the media was withdrawn and the cells

were dissolved in RNA lysis buffer and RNA was prepared as described, above, in Section 6.1.

Lipoproteins For oxidation, human LDL (Sigma) was first diluted to 1 mg/ml with PBS and then dialyzed against 5 PBS at 4°C overnight. LDL was then diluted to 0.3 mg/ml with PBS. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ was then added to 5uM final concentration, and the solution was incubated in a T flask in a 37°C incubator for 24 hr. LDL solution was then dialyzed at 4°C against 0.15M NaCl/0.3mM EDTA for 2 days with several 10 changes, before being removed and concentrated using an Amicon spin column by spinning for 1 hr. 4000 RPM at 4°C.

For acetylation, 1 ml of 5 mg/ml LDL was added to 1 ml of a saturated solution of NaOAc in a 15 ml tube on ice on a shaker at 4°C. 8 μl of acetic anhydride was added 2 μl at 15 a time over 1 hr. LDL was then dialyzed for 48 hr. against 0.15M NaCl/0.3 mM EDTA at 4°C for 48 hr. with several changes. Final concentrations of derivatized LDL's were determined by comparing to a dilution curve of native LDL analyzed at OD_{280} , with 0.15M NaCl/0.3mM EDTA used as diluent 20 in all cases.

6.1.2. ANALYSIS OF PARADIGM MATERIAL

Differential Display:

Removal of DNA: The RNA pellet was resuspended in H_2O 25 and quantified by spectrophotometry at OD_{260} . Approximately half of the sample was then treated with DNase I to remove contaminating chromosomal DNA. RNA was amplified by PCR using the following procedure. 50 μl RNA sample (10-20 μg), 5.7 μl 10x PCR buffer (Perkin-Elmer/Cetus), 1 μl RNase 30 inhibitor (40 units/ μl) (Boehringer Mannheim, Germany) were mixed together, vortexed, and briefly spun. 2 μl DNase I (10 units/ μl) (Boehringer Mannheim) was added to the reaction which was incubated for 30 min. at 37°C. The total volume was brought to 200 μl with DEPC H_2O , extracted once with 35 phenol/chloroform, once with chloroform, and precipitated by adding 20 μl 3M NaOAc, pH 4.8, (DEPC-treated), 500 μl absolute ETOH and incubating for 1 hour on dry ice or -20°C

overnight. The precipitated sample was centrifuged for 15 min., and the pellet was washed with 70% ETOH. The sample was re-centrifuged, the remaining liquid was aspirated, and the pellet was resuspended in 100 μ l H₂O. The concentration of RNA was measured by reading the OD₂₆₀.

First strand cDNA synthesis: For each RNA sample duplicate reactions were carried out in parallel. 400 ng RNA plus DEPC H₂O in a total volume of 10 μ l were added to 4 μ l T₁₁XX reverse primer (10 μ M) (Operon). The specific primers used in each experiment are provided in the Description of the Figures in Section 4, above. The mixture was incubated at 70°C for 5 min. to denature the RNA and then placed at r.t. 26 μ l of reaction mix containing the following components was added to each denatured RNA/primer sample: 8 μ l 5x First Strand Buffer (Gibco/BRL, Gaithersburg, MD), 4 μ l 0.1M DTT (Gibco/BRL), 2 μ l RNase inhibitor (40 units/ μ l) (Boehringer Mannheim), 4 μ l 200 μ M dNTP mix, 6 μ l H₂O, 2 μ l Superscript reverse transcriptase (200 units/ μ l) (Gibco/BRL). The reactions were mixed gently and incubated for 30 min. at 42°C. 60 μ l of H₂O (final volume = 100 μ l) were then added and the samples were denatured for 5 min. at 85°C and stored at -20°C.

PCR reactions: 13 μ l of reaction mix was added to each tube of a 96 well plate on ice. The reaction mix contained 6.4 μ l H₂O, 2 μ l 10x PCR Buffer (Perkin-Elmer), 2 μ l 20 μ M dNTP's, 0.4 μ l ³⁵S dATP (12.5 μ Ci/ μ l; 50 μ Ci total) (Dupont/NEN), 2 μ l forward (for-) primer (10 μ M) (Operon), and 0.2 μ l AmpliTaq Polymerase (5 units/ μ l) (Perkin-Elmer). Next, 2 μ l of reverse (rev-) primer (T₁₁XX, 10 μ M) were added to the side of each tube followed by 5 μ l of cDNA also to the sides of the tubes, which were still on ice. The specific primers used in each experiment were as follows:

fchd602: rev-T₁₁XC and for-GTGAGGCGTC
fchd605: rev-T₁₁XC and for-TGGACCGGTG

Tubes were capped and mixed, and brought up to 1000 RPM in a centrifuge then returned immediately to ice. The PCR machine (Perkin-Elmer 9600) was programmed for differential display as follows:

5	94°C	2 min.
	*94°C	15 sec.
	*40°C	2 min.
*	= X40	*ramp 72°C
		1 min.
	*72°C	30 sec.
10	72°C	5 min.
	4°C	hold

When the PCR machine reached 94°C, the plate was removed from ice and placed directly into the Perkin-Elmer 9600 PCR machine. Following PCR, 15 µl of loading dye, containing 80% formamide, 10 mM EDTA, 1 mg/ml xylene cyanol, 1 mg/ml bromphenol blue were added. The loading dye and reaction were mixed, incubated at 85°C for 5 min., cooled on ice, centrifuged, and placed on ice. Approximately 4 µl from each tube were loaded onto a prerun (60V) 6% acrylamide gel. The gel was run at approximately 80V until top dye front was about 1 inch from bottom. The gel was transferred to 3MM paper (Whatman Paper, England) and dried under vacuum. Bands were visualized by autoradiography.

25

Band isolation and amplification: Differentially expressed bands were excised from the dried gel with a razor blade and placed into a microfuge tube with 100 µl H₂O and heated at 100°C for 5 min., vortexed, heated again to 100°C for 5 min., and vortex again. After cooling, 100 µl H₂O, 20 µl 3M NaOAc, 1 µl glycogen (20 mg/ml), and 500 µl ethanol were added and chilled. After centrifugation, the pellet was washed and resuspended in 10 µl H₂O.

The isolated differentially expressed bands were then amplified by PCR using the following reaction conditions:

58	µl	H ₂ O
10	µl	10x PCR Buffer

	10	μ l	200 μ M dNTP's
	10	μ l	10 μ M reverse primer
	10	μ l	10 μ M forward primer
	1.5	μ l	amplified band
5	0.5	μ l	AmpliTaq polymerase (5 units/ μ l) (Perkin Elmer)

PCR was performed using the program described in this Section, above, for differential display. After PCR, glycerol loading dyes were added and samples were loaded onto a 2% preparative TAE/Biogel (Bio101, La Jolla, CA) agarose gel and eluted. Bands were then excised from the gel with a razor blade and vortexed for 15 min. at r.t., and purified using the Mermaid kit from Bio101 by adding 3 volumes of Mermaid high salt binding solution and 8 μ l of resuspended glassfog in a microfuge tube. Glassfog was then pelleted, washed 3 times with ethanol wash solution, and then DNA was eluted twice in 10 μ l at 50°C.

Subcloning: The TA cloning kit (Invitrogen, San Diego, CA) was used to subclone the amplified bands. The ligation reaction typically consisted of 4 μ l sterile H₂O, 1 μ l ligation buffer, 2 μ l TA cloning vector, 2 μ l PCR product, and 1 μ l T4 DNA ligase. The volume of PCR product can vary, but the total volume of PCR product plus H₂O was always 6 μ l. Ligations (including vector alone) were incubated overnight at 12°C before bacterial transformation. TA cloning kit competent bacteria (INV α F': *endA1*, *recA1*, *hsdR17(r-k, m+k)*, *supE44*, λ^- , *thi-1*, *gyrA*, *relA1*, ϕ 80lacZ α M15 Δ (*lacZYA-argF*), *deoR+*, F') were thawed on ice and 2 μ l of 0.5 M β -mercaptoethanol were added to each tube. 2 μ l from each ligation were added to each tube of competent cells (50 μ l), mixed without vortexing, and incubated on ice for 30 min. Tubes were then placed in 42°C bath for exactly 30 sec., before being returned to ice for 2 min. 450 μ l of SOC media (Sambrook et al., 1989, *supra*) were then added to each tube which were then shaken at 37°C for 1 hr. Bacteria were then pelleted, resuspended in ~200 μ l SOC and plated on Luria

broth agar plates containing X-gal and 60 µg/ml ampicillin and incubated overnight at 37°C. White colonies were then picked and screened for inserts using PCR.

A master mix containing 2 µl 10x PCR buffer, 1.6 µl 5 2.5 mM dNTP's, 0.1 µl 25 mM MgCl₂, 0.2 µl M13 reverse primer (100 ng/µl), 0.2 µl M13 forward primer (100 ng/µl), 0.1 µl AmpliTaq (Perkin-Elmer), and 15.8 µl H₂O was made. 40 µl of the master mix were aliquoted into tubes of a 96 well plate, and whole bacteria were added with a pipette tip prior to 10 PCR. The PCR machine (Perkin-Elmer 9600) was programmed for insert screening as follows:

	94°C	2 min.
	*94°C	15 sec.
	*47°C	2 min.
15	* = X35	*ramp 72°C 30 sec.
	*72°C	30 sec.
	72°C	10 min.
	4°C	hold

Reaction products were eluted on a 2% agarose gel and 20 compared to vector control. Colonies with vectors containing inserts were purified by streaking onto LB/Amp plates. Vectors were isolated from such strains and subjected to sequence analysis, using an Applied Biosystems Automated Sequencer (Applied Biosystems, Inc. Seattle, WA).

25

Northern analysis: Northern analysis was performed to confirm the differential expression of the genes corresponding to the amplified bands. The probes used to detect mRNA were synthesized as follows: typically 2 µl 30 amplified band (~30 ng), 7 µl H₂O, and 2 µl 10x Hexanucleotide mix (Boehringer-Mannheim) were mixed and heated to 95°C for 5 min., and then allowed to cool on ice. The volume of the amplified band can vary, but the total volume of the band plus H₂O was always 9 µl. 3 µl dATP/dGTP/dTTP mix (1:1:1 of 35 0.5 mM each), 5 µl α³²P dCTP 3000 Ci/mM (50 µCi total) (Amersham, Arlington Heights, IL), and 1 µl Klenow (2 units) (Boehringer-Mannheim) were mixed and incubated at 37°C.

After 1 hr., 30 μ l TE were added and the reaction was loaded onto a Biospin-6™ column (Biorad, Hercules, CA), and centrifuged. A 1 μ l aliquot of eluate was used to measure incorporation in a scintillation counter with scintillant to 5 ensure that 10⁶cpm/ μ l of incorporation was achieved.

The samples were loaded onto a denaturing agarose gel. A 300 ml 1% gel was made by adding 3 g of agarose (SeaKem™ LE, FMC BioProducts, Rockland, ME) and 60 ml of 5x MOPS buffer to 210 ml sterile H₂O. 5x MOPS buffer (0.1M MOPS (pH 10 7.0), 40 mM NaOAc, 5mM EDTA (pH 8.0)) was made by adding 20.6 g of MOPS to 800 ml of 50mM NaOAc (13.3 ml of 3M NaOAc pH 4.8 in 800 ml sterile H₂O); then adjusting the pH to 7.0 with 10M NaOH; adding 10 ml of 0.5M EDTA (pH8.0); and adding H₂O to a final volume of 1L. The mixture was heated until melted, 15 then cooled to 50°C, at which time 5 μ l ethidium bromide (5mg/ml) and 30 ml of 37% formaldehyde of gel were added. The gel was swirled quickly to mix, and then poured immediately.

2 μ g RNA sample, 1x final 1.5x RNA loading dyes (60% 20 formamide, 9% formaldehyde, 1.5X MOPS, .075% XC/BPB dyes) and H₂O were mixed to a final volume of 40 μ l. The tubes were heated at 65°C for 5 min. and then cooled on ice. 10 μ g of RNA MW standards (New England Biolabs, Beverly, MA) were also denatured with dye and loaded onto the gel. The gel was run 25 overnight at 32V in MOPS running buffer.

The gel was then soaked in 0.5 μ g/ml Ethidium Bromide for 45 min., 50 mM NaOH/0.1 M NaCl for 30 min., 0.1 M Tris pH 8.0 for 30 min., and 20x SSC for 20 min. Each soaking step was done at r.t. with shaking. The gel was then photographed 30 along with a fluorescent ruler before blotting with Hybond-N membrane (Amersham), according to the methods of Sambrook et al., 1989, *supra*, in 20x SSC overnight.

Northern blot hybridizations were carried out as follows: for pre-hybridization, the blot was placed into 35 roller bottle containing 10 ml of rapid-hyb solution (Amersham), and placed into 65°C incubator for at least 1 hr. For hybridization, 1x10⁷ cpm of the probe was then heated to

95°C, chilled on ice, and added to 10 ml of rapid-hyb solution. The prehybridization solution was then replaced with probe solution and incubated for 3 hr at 65°C. The following day, the blot was washed once for 20 min. at r.t. in 2x SSC/0.1% SDS and twice for 15 min. at 65°C in 0.1x SSC/0.1% SDS before being covered in plastic wrap and put down for exposure.

RT-PCR Analysis: RT-PCR was performed to detect differentially expressed levels of mRNA from the genes corresponding to amplified bands. First strand synthesis was conducted by mixing 20 μ l DNase RNA (~2 μ g), 1 μ l oligo dT (Operon) (1 μ g), and 9.75 μ l H₂O. The samples were heated at 70°C for 10 min., and then allowed to cool on ice. 10 μ l first strand buffer (Gibco/BRL), 5 μ l 0.1M DTT, 1.25 μ l 20 mM dNTP's (500 μ M final), 1 μ l RNasin (40 units/ μ l) (Boehringer Mannheim), and 2 μ l Superscript Reverse Transcriptase (200 units/ μ l) (Gibco/BRL) were added to the reaction, incubated at 42°C for 1 hr., and then placed at 85°C for 5 min., and stored at -20°C.

PCR was performed on the reverse transcribed samples. Each reaction contained 2 μ l 10x PCR buffer, 14.5 μ l H₂O, 0.2 μ l 20 mM dNTP's (200 μ M final), 0.5 μ l 20 μ M forward primer (0.4 μ M final), 0.5 μ l 20 μ M reverse primer (0.4 μ M final), 0.3 μ l AmpliTaq polymerase (Perkin-Elmer/Cetus), 2 μ l cDNA dilution or positive control (~40 pg). The specific primers used in each experiment are provided in the Description of the Figures in Section 4, above. Samples were placed in the PCR 9600 machine at 94°C (hot start), which was programmed as follows:

30	94°C	2 min. (samples loaded)
	*94°C	45 sec.
* = 35x	*55°C	45 sec.
	*72°C	2 min.
	72°C	5 min.
35	4°C	hold

Reactions were carried out on cDNA dilution series and tubes were removed at various cycles from the machine during

72°C step. Reaction products were eluted on a 1.8% agarose gel and visualized with ethidium bromide.

Gene Retrieval: Amplified sequences, which
5 contained portions of the genes, were subcloned and then used individually to retrieve a cDNA encoding the corresponding gene. Probes were prepared by isolating the subcloned insert DNA from vector DNA, and labeling with ³²P as described above in Section 6.1.2. Labeled insert DNA containing fchd602
10 sequences was used to probe a cDNA library prepared from human macrophage cell line U937. Labeled insert DNA containing fchd605 sequences was used to probe a cDNA library prepared from human primary blood monocytes. The cDNA
15 libraries were prepared and screened according to methods routinely practiced in the art (see Sambrook et al., 1989, *supra*). Plaques from the libraries that were detected by the probes were isolated and the cDNA insert within the phage vector was sequenced.

The RACE procedure kit was used either as an
20 alternative to cDNA library screening, or, when the cDNA library did not yield a clone encoding the full-length gene, to obtain adjacent sequences of the gene. The procedure was carried out according to the manufacturer's instructions (Clontech, Palo Alto, CA; see also: Chenchik, et al., 1995,
25 CLONTECHniques (X) 1: 5-8; Barnes, 1994, Proc. Natl. Acad. Sci. USA 91: 2216-2220; and Cheng et al., Proc. Natl. Acad. Sci. USA 91: 5695-5699). Primers were designed based either on amplified sequences, or on sequences obtained from isolates from the cDNA libraries. Template mRNA for fchd605
30 was isolated from human primary blood monocytes.

6.1.3. CHROMOSOMAL LOCALIZATION OF TARGET GENES

Once the nucleotide sequence has been determined, the presence of the gene on a particular chromosome is detected.
35 Oligonucleotide primers based on the nucleotide sequence of the target gene are used in PCR reactions using individual human chromosomes as templates. Individual samples of each

the twenty-three human chromosomes are commercially available (Coriel Institute for Medical Research, Camden, NJ). The chromosomal DNA is amplified according to the following conditions: 10ng chromosomal DNA, 2 μ l 10x PCR buffer, 1.6 μ l 5 2.5mM dNTP's, 0.1 μ l 25mM MgCl₂, 0.2 μ l reverse primer (100ng/ μ l), 0.2 μ l forward primer (100ng/ μ l), 0.1 μ l Taq polymerase, and 15.8 μ l H₂O. Samples are placed in the PCR 9600 machine at 94°C (hot start), which is programmed as follows:

10		94°C	2 min. (samples loaded)
		*94°C	20 sec.
	* = 35x	*55°C	30 sec.
		*72°C	30 sec.
15		72°C	5 min.
		4°C	hold

6.2. RESULTS

Differential display was performed on monocytes 20 treated with oxidized LDL and untreated monocytes. Bands corresponding to fchd602 and fchd605 were detected as up-regulated by oxidized LDL, as compared with the untreated monocytes. The up-regulation was confirmed by northern blot analysis.

25 The fchd602 gene produced a 2.5kb mRNA that was up-regulated after 5 hours of treatment with oxidized LDL, minimally oxidized LDL, and lysophosphatidylcholine. No message was detected in untreated or native LDL treated control monocytes. The amplified DNA sequence was used to 30 recover a cDNA of approximately 875 bp comprising an open reading frame encoding approximately 182 amino acids. The DNA sequence and encoded amino acid sequence of this cDNA from the fchd602 gene is shown in FIG.4. The open reading frame has 88% sequence similarity to the rat Cl-6 gene, which 35 is induced in regenerating rat liver, is insulin inducible, and also contains multiple transmembrane domains (Diamond, R.H., et al., 1993, J. Biol. Chem. 268: 15185-15192).

The fchd605 gene produced a 1.5kb mRNA that is up-regulated after 5 hours treatment with oxidized LDL, and to a lesser degree with native LDL, as compared to untreated monocytes. The amplified DNA was sequenced and used to
5 recover a cDNA of approximately 2.2kb, which was sequenced to reveal a partial open reading frame of approximately 258 bp, encoding approximately 86 amino acids. The DNA sequence and encoded amino acid sequence from the fchd605 gene is shown in FIG.5. The sequence has similarity to the mouse gly96 gene,
10 which encodes a cytokine inducible glycosylated protein expressed in mouse lung, testes, and uterus.

7. EXAMPLE: IDENTIFICATION OF GENES DIFFERENTIALLY EXPRESSED IN RESPONSE TO PARADIGM D: ENDOTHELIAL CELL SHEAR STRESS

15 According to the invention, differential display was used to detect genes that are differentially expressed in endothelial cells that were subjected to fluid shear stress in vitro. Shear stress is thought to be responsible for the prevalence of atherosclerotic lesions in areas of unusual
20 circulatory flow. Using the method of Paradigm D, three novel DNA sequences were identified.

The fchd531 gene is down-regulated in endothelial cells under both turbulent and laminar shear stress, as compared to the static control. The fchd531 gene encodes a
25 novel 570 amino acid polypeptide, and has 94% sequence similarity to the mouse penta zinc finger gene (Pzf), which has not been published, but is contained in the GenBank sequence data base under accession no U05343.

30 The fchd540 gene is up-regulated in endothelial cells under laminar shear stress, but is not up-regulated by IL-1 treatment. The fchd540 gene encodes a novel intracellular protein which has sequence similarity to the Drosophila Mad protein (Sekelsky et al., 1995, Genetics 139: 1347-1358).

35 The fchd545 gene is down-regulated in endothelial cells under laminar shear stress as compared to endothelial cells under turbulent shear stress and static control

endothelial cells. The fchd545 gene encodes an 848 amino acid polypeptide which has 73% sequence similarity to the human Voltage-dependent Anion Channel protein (Blachly-Dyson, E., et al., 1993, J. Biol. Chem. 268: 1835-1841.). The fchd545 gene is also expressed in the human heart, smooth muscles, and testes.

The up-regulation of the fchd540 gene and down-regulation of the fchd531 and fchd545 genes in shear stressed endothelial cells provides a fingerprint for the study of cardiovascular diseases, including but not limited to atherosclerosis, ischemia/reperfusion, hypertension, and restenosis. The fact that one of these genes, fchd540, is not up-regulated under Paradigm C (IL-1 induction) provides an extremely useful means of distinguishing and targeting physiological phenomena specific to shear stress.

Furthermore, as a transmembrane protein, the fchd545 gene product can be readily accessed or detected on the endothelial cell surface by other compounds. It provides, therefore, an excellent target for detection of cardiovascular disease states in diagnostic systems, as well as in the monitoring of the efficacy of compounds in clinical trials. Furthermore, the extracellular domains of this gene product provide targets which allow for designing especially efficient screening systems for identifying compounds that bind to them. Such compounds can be useful in treating cardiovascular disease by modulating the activity of the transmembrane gene product.

7.1. MATERIALS AND METHODS

Primary cultures of HUVEC's were established from normal term umbilical cords as described (In Progress in Hemostasis and Thrombosis, Vol. 3, P. Spaet, editor, Grune & Stratton Inc., New York, 1-28). Cells were grown in 20% fetal calf serum complete media (1989, J. Immunol. 142: 2257-2263) and passaged 1-3 times before shear stress induction.

For induction, second passage HUVEC's were plated on tissue culture-treated polystyrene and subjected to 10 dyn/cm²

laminar flow for 1 and 6 hr. as described (1994, J. Clin. Invest. 94: 885-891) or 3-10 dyn/cm² turbulent flow as previously described (1986 Proc. Natl. Acad. Sci. U.S.A. 83: 2114-2117). RNA was isolated as described, above, in Section 5 6.1. Differential display, Northern analysis, RT-PCR, subcloning, and DNA sequencing were carried out as described, above, in Section 6.1.2. Specific primers used in differential display were as follows:

- fchd531: for-T₁₁XA and rev-AGACGTCCAC
- 10 fchd540: for-T₁₁XA and rev-ACTTCGCCAC
- fchd545: for-T₁₁XC and rev-TCGGACGTGA

Amplified sequences, which contained portions of the genes, were subcloned and then used individually to retrieve 15 a cDNA encoding the corresponding gene. Probes were prepared by isolating the subcloned insert DNA from vector DNA, and labeling with ³²P as described above in Section 6.1.2. Labeled insert DNA was used to probe cDNA library prepared from shear stress induced endothelial cells. The library was 20 prepared and probed using methods routinely practiced in the art (see Sambrook et al., 1989, supra). Plaques from the libraries that were detected by the probes were isolated and the cDNA insert within the phage vector was sequenced.

The RACE procedure kit was used either as an 25 alternative to cDNA library screening, or, when the cDNA library did not yield a clone encoding the full-length gene, to obtain adjacent sequences of the gene. The procedure was carried out according to the manufacturer's instructions (Clontech, Palo Alto, CA; see also: Chenchik, et al., 1995, 30 CLONTECHniques (X) 1: 5-8; Barnes, 1994, Proc. Natl. Acad. Sci. USA 91: 2216-2220; and Cheng et al., Proc. Natl. Acad. Sci. USA 91: 5695-5699). Primers were designed based either on amplified sequences, or on sequences obtained from isolates from the cDNA libraries. Template mRNA was isolated 35 from shear stressed HUVEC's.

Northern blot analysis of RNA extracted from various human organs and tissues was performed using commercially available pre-blotted filters (Clontech, Palo Alto, CA).

5 7.2. RESULTS

An amplified fchd531 fragment obtained from differential display was subcloned and sequenced, and used to obtain a 1.9 kb cDNA containing the entire fchd531 coding region. The DNA sequence and encoded amino acid sequence of
10 the novel fchd531 gene is shown in FIG.1. The fchd531 gene encodes a 570 amino acid polypeptide, and has 94% sequence similarity to the mouse penta zinc finger gene (Pzf) (GenBank accession number U05343). Northern analysis of HUVEC's which were subjected turbulent and laminar shear stress
15 demonstrated that the fchd531 gene produces an approximately 5 kb message which is down-regulated under laminar shear stress, but not turbulent shear stress, compared with the static control.

The fchd540 gene was detected as an up-regulated
20 message under shear stress. The amplified fragment was used to probe a Northern blot containing samples from HUVECs treated with laminar shear stress. A 4.4 kb fchd540 mRNA is up-regulated after 6 hours treatment with laminar shear stress. The fchd540 gene is not induced by IL-1 by the
25 method of Paradigm C, (Section 5.1.1.5, above). The amplified fragment was sequenced and used to obtain a 2.7 kb cDNA containing the entire fchd540 coding region. The DNA sequence and encoded amino acid sequence from the fchd540 gene is shown in FIG.2. The fchd540 gene encodes a 426 amino
30 acid polypeptide and has sequence similarity to the *Drosophila* Mad gene (Sekelsky et al., 1995, Genetics 139: 1347-1358).

The fchd545 gene was detected as a down-regulated message under shear stress. Northern analysis revealed that
35 the fchd545 gene produces a 1.4kb message which is down regulated by turbulent shear stress, but not by laminar shear stress, as compared with static control. The amplified

fragment was sequenced and used to isolate a 1.4kb cDNA containing the complete fchd545 coding sequence. The DNA sequence and encoded amino acid sequence of the fchd545 gene is shown in FIG.3. The fchd545 gene encodes a 283 amino acid polypeptide which has 73% sequence similarity to the human Voltage-dependent Anion Channel (Blachly-Dyson, E., et al., 1993, J. Biol. Chem. 268: 1835-1841). Northern analysis of a commercially available (Clontech, Palo Alto, California) northern blot revealed that the fchd545 gene is expressed in human heart, smooth muscle, and testes.

8. EXAMPLE: USE OF GENES UNDER PARADIGM A AS SURROGATE MARKERS IN CLINICAL TRIALS

According to the invention, the fingerprint profile derived from any of the paradigms described in Sections 5.1.1.1 through 5.1.1.6 may be used to monitor clinical trials of drugs in human patients. The fingerprint profile, described generally in Section 5.5.4, above, indicates the characteristic pattern of differential gene regulation corresponding to a particular disease state. Paradigm A, described in Section 5.1.1.1, and illustrated in the example in Section 6, above, for example, provides the fingerprint profile of monocytes under oxidative stress. The target genes, therefore, serve as surrogate markers by giving an indicative reading of the physiological response of monocytes to the uptake of oxidized LDL. Accordingly, the influence of anti-oxidant drugs on the oxidative potential may be measured by performing differential display on the monocytes of patients undergoing clinical tests.

30

8.1. TREATMENT OF PATIENTS AND CELL ISOLATION

Test patients may be administered compounds suspected of having anti-oxidant activity. Control patients may be given a placebo.

35 Blood may be drawn from each patient after a 12 hour period of fasting and monocytes may be purified as described, above, in Section 7.1.1. RNA may be isolated as described in

Section 6.1.1, above. Primers may then be designed for amplification based on the DNA sequence of target genes identified as up-regulated, such as fchd602 and fchd605, or down-regulated under Paradigm A.

5

8.2. ANALYSIS OF SAMPLES

RNA may be subjected to differential display analysis as described in Section 6.1.2, above. A decrease in the physiological response state of the monocytes is indicated by
10 a decreased intensity of those bands corresponding to fchd602 and fchd605, which were up-regulated by oxidized LDL under Paradigm A, as described in Section 6.2, above.

9. EXAMPLE: IMAGING OF A CARDIOVASCULAR DISEASE CONDITION

15 According to the invention, differentially expressed gene products which are localized on the surface of affected tissue may be used as markers for imaging the diseased or damaged tissue. Conjugated antibodies that are specific to the differentially expressed gene product may be administered
20 to a patient or a test animal intravenously. This method provides the advantage of allowing the diseased or damaged tissue to be visualized non-invasively.

For the purposes of illustration, this method is described in detail for the fchd602 gene product. The
25 principles and techniques can be applied to any transmembrane target gene product, including, for example, the fchd545 gene product.

9.1. MONOCLONAL CONJUGATED ANTIBODIES

30 The differentially expressed surface gene product, such as the fchd602 gene product, is expressed in a recombinant host and purified using methods described in Section 5.4.2, above. Preferably, a protein fragment comprising one or more of the extracellular domains of the
35 fchd602 product is produced. Once purified, it is be used to produce $F(ab')_2$ or Fab fragments, as described in Section 5.4.3, above. These fragments are then labelled with

technetium-99m (^{99m}Tc) using a conjugated metal chelator, such as DTPA as described in section 5.8.3, above.

9.2. ADMINISTRATION AND DETECTION OF IMAGING AGENTS

5 Labeled MAb may be administered intravenously to a patient being diagnosed for atherosclerosis, restenosis, or ischemia/reperfusion. Sufficient time is allowed for the detectably-labeled antibody to localize at the diseased or damaged tissue site (or sites), and bind to the fchd602 gene
10 product. The signal generated by the label is detected by a photoscanning device. The detected signal is then converted to an image of the tissue, revealing cells, such as monocytes, in which fchd602 gene expression is up-regulated.

15 10. POLYCLONAL ANTIBODIES TO TARGET GENE PEPTIDE SEQUENCES

Peptide sequences corresponding to the indicated amino sequences of cDNAs were selected and submitted to Research Genetics (Huntsville, AL) for synthesis and antibody
20 production. Peptides were modified as described (Tam, J.P., 1988, Proc. Natl. Acad. Sci. USA 85: 5409-5413; Tam, J.P., and Zavala, F., 1989, J. Immunol. Methods 124: 53-61; Tam, J.P., and Lu, Y.A., 1989, Proc. Natl. Acad. Sci. USA 86: 9084-9088), emulsified in an equal volume of Freund's adjuvant and injected into rabbits at 3 to 4 subcutaneous
25 dorsal sites for a total volume of 1.0 ml (0.5 mg peptide) per immunization. The animals were boosted after 2 and 6 weeks and bled at weeks 4, 8, and 10. The blood was allowed to clot and serum was collected by centrifugation.

The peptides used are summarized below:

30

fchd545 Peptide Antigens

Name	Position	Sequence
fchd545.1	48-63	YTDTGKASGNLETKYK
fchd545.2	107-121	TGKKSGKLKASYKRD

35

11. EXAMPLE: THE RCHD534 AND FCHD540 GENE PRODUCTS INTERACT

The novel fchd540 gene and its nucleotide sequence is described in Section 7, above. The fchd540 gene shares
5 homology with the *Drosophila* Mad gene. The rchd534 gene (described in Applicant's co-pending PCT Application Publication No. WO 96/24604, which is incorporated by reference in its entirety herein) is another gene that is up-regulated in endothelial cells by shear stress. The DNA and
10 encoded amino acid sequence of the rchd534 gene is shown in FIG.6. The rchd534 gene was deposited in the Agricultural Research Service Culture Collection (NRRL) in microorganism FCHD534 on June 6, 1995 and assigned the NRRL Accession No. B-21459. The rchd534 gene also shares homology with the
15 *Drosophila* Mad gene. Mad genes have been shown to play a role in the TGF- β signalling pathway (Sekelsky et al., 1995, Genetics 139: 1347-1358; Chen et al., 1996, Nature 383: 691-696; Serra, et al., 1996, Nature Medicine 2: 390-391). TGF- β signalling is considered to be beneficial to atherosclerosis and restenosis (Border et al., 1995, Nature Medicine 1: 1000;
20 Grainger, et al., 1995, Nature Medicine 1: 1067-1073; Kojima, et al., 1991, J. Cell Biol. 113: 1439-1445; Nikol, et al., 1992, J. Clin. Invest. 90: 1582-1592).

The data described below demonstrate that the rchd534 and
25 fchd540 proteins interact with one another; and this interaction may lead to the inhibition of TGF- β signalling. Furthermore, the expression of these two genes, as described below, is specific to endothelial cells. Because these two genes 1) are both expressed specifically in endothelial
30 cells, 2) are both up-regulated in endothelial cells under certain conditions, 3) encode MAD proteins that interact with one another in endothelial cells, and 4) inhibit TGF- β signalling (which is considered to be beneficial to atherosclerosis), rchd534 and fchd540 proteins are attractive
35 targets for therapeutic intervention in cardiovascular disease. In particular, treatment regimens that inhibit the

interaction or activity of the rchd534 and fchd540 proteins can be beneficial for the treatment cardiovascular disease.

Further analyses demonstrated that the rchd534 protein interacts with itself to form a homodimer. Thus, treatment 5 regimens that inhibit the interaction of the rchd534 protein with itself can be beneficial for the treatment cardiovascular disease.

In addition, the analyses described below demonstrated novel interactions of both the rchd534 and fchd540 proteins 10 with other proteins known to be involved in the TGF- β signalling pathway. The protein members of the TGF- β signalling pathway tested included MADR1 (Hoodless et al., 1996, Cell 85:489-500), MADR2 (Eppert et al., 1996, Cell 86: 543-552), DPC4 (Raftery et al., 1988, Genetics 139: 241-254), 15 T β RI, TSR1, ActRIb, ALK3, and ALK6 (Wieser et al., 1995, EMBO J. 14: 2199-2208). For example, the rchd534 protein interacts strongly in endothelial cells with MADR1, MADR2, DPC4, and weakly in 293 (human embryonic kidney) cells with activated forms of receptors T β RI and ActRI. The fchd540 20 protein interacts strongly in 293 cells with activated forms of receptors T β RI and ALK6.

In the absence of transfected rchd543 and fchd540 genes, transfected MADR1 or transfected MADR2 mediated a 20-fold induction of a TGF- β inducible promoter in BAECs. Co- 25 expression of either transfected rchd534 or transfected rchd540 in this system eliminated the induction, and also prevented the localization of MADR2 in the nucleus in response to TGF- β signalling. Therefore, treatment regimens that inhibit the interaction of the rchd534 and fchd540 30 proteins with other proteins involved in the TGF- β pathway also can be beneficial for the treatment cardiovascular of disease. As described above, the expression of rchd534 and fchd540 is specific, within arterial tissue, to endothelial cells. Accordingly, the rchd534 and rchd540 genes may be 35 targets for intervention in a variety of inflammatory and fibroproliferative disorders that involve endothelial cells,

including, but not limited to, cancer angiogenesis, inflammation, and fibrosis.

11.1. Materials and Methods

5

11.1.1. Yeast Strains, Media, and Microbiological Techniques

Standard yeast media including synthetic complete medium lacking L-leucine, L-tryptophan, and L-histidine were prepared and yeast genetic manipulations were performed as described (Sherman, 1991, Meth. Enzymol., 194:3-21). Yeast transformations were performed using standard protocols (Gietz et al., 1992, Nucleic Acids Res., 20:1425. Ito et al., 1983, J. Bacteriol., 153:163-168). Plasmid DNAs were isolated from yeast strains by a standard method (Hoffman and Winston, 1987, Gene, 57:267-272).

10

15

11.1.2. Plasmid and Yeast Strain Construction

The coding region of human fchd540 was amplified by PCR and cloned in frame into pGBT9 (Bartel et al., 1993, Cellular Interactions in Development. pp. 153-159) resulting in plasmid pGBT9-fchd540. pGBT9-fchd540 was transformed into two-hybrid screening strain HF7c and one resulting transformant was designated TB35.

20

25

11.1.3. Two-Hybrid Screening

Two-hybrid screening was carried out essentially as described (Bartel et al., 1993, *supra*) using TB35 as the recipient strain and a human breast two-hybrid library.

30

11.1.4. Paper Filter Beta-Galactosidase Assays

The paper filter beta-galactosidase (beta-gal) assay was performed essentially as previously described (Brill et al., 1994, Mol. Biol. Cell 5: 297-312).

35

11.2 Results

11.2.1. Strong physical interaction of rchd534 and fchd540 measured by two-hybrid assay

The fchd540 coding sequence was amplified by PCR and cloned
5 into pGBT9 creating a GAL4 DNA-binding domain-fchd540 fusion
gene. The screening strain HF7c was transformed with this
construct. The rchd534 coding sequence was cloned into
pGAD424 (Bartel et al., 1993, *supra*) creating a GAL4
transcriptional activation domain-rchd534 fusion gene, which
10 was then used to transform strain Y187.

Yeast expression plasmids encoding the GAL4 DNA-binding
domain either alone or fused in frame to fchd540, rchd534,
Drosophila MAD, DPC4, or p53 were transformed into MAT α two-
hybrid screening strain HF7c. Yeast expression plasmids
15 encoding the GAL4 transcriptional activation domain alone and
GAL4 activation domain fusions to rchd534 and SV40 were
transformed into MAT α two-hybrid screening strain Y187. p53
and SV40 interact with each other and should not interact
with the experimental proteins. The HF7c transformants were
20 propagated as stripes on semisolid synthetic complete medium
lacking L-tryptophan and the Y187 transformants were grown as
stripes on semisolid synthetic complete medium lacking L-
leucine. Both sets of stripes were replica plated in the
form of a grid onto a single rich YPAD plate and the haploid
25 strains of opposite mating types were allowed to mate
overnight at 30°C. The yeast strains on the mating plate
were then replica plated to a synthetic complete plate
lacking L-leucine and L-tryptophan to select for diploids and
incubated at 30°C overnight. Diploid strains on the
30 synthetic complete plate lacking L-leucine and L-tryptophan
were replica plated to a synthetic complete plate lacking L-
leucine, L-tryptophan, and L-histidine to assay HIS3
expression and a paper filter on a synthetic complete plate
lacking L-leucine and L-tryptophan. The next day the paper
35 filter was subjected to the paper filter beta-galactosidase
assay to measure expression of the lacZ reporter gene. HIS3

expression was scored after 3 days of growth at 30°C. The results are shown in Table 3.

The rchd534 fish protein was found to interact strongly with the fchd540 bait protein and not to interact with the rchd534, MAD, DPC4, p53, and GAL4 DNA binding domain bait proteins. This result demonstrated that rchd534 and fchd540 strongly physically interact with each other with significant specificity.

10 11.2.2. Identification of proteins that physically interact with fchd540

The fchd540 coding sequence was amplified by PCR and cloned into pGBT9 (Bartel et al., 1993, supra) creating a GAL4 DNA-binding domain-fchd540 fusion gene. HF7c was transformed with this construct resulting in strain TB35. TB35 grew on synthetic complete medium lacking L-tryptophan but not on synthetic complete medium lacking L-tryptophan and L-histidine demonstrating that the GAL4 DNA-binding domain-fchd540 fusion does not have intrinsic transcriptional activation activity.

20 TB35 was transformed with the human breast two-hybrid library and 5 million transformants were obtained. The transformants were plated on synthetic complete medium lacking L-leucine, L-tryptophan, and L-histidine and yeast colonies that both grew on synthetic complete medium lacking L-leucine, L-tryptophan, and L-histidine and expressed the beta-galactosidase reporter gene were identified. The 30 strains with the strongest beta-galactosidase induction were characterized. Library plasmids were isolated from these strains, and the 5' ends of all of the cDNA inserts were sequenced.

 11.2.3. Retransformation and specificity testing of tchv03A and tchvR4A

35 Two of the plasmids that encoded the strongest interactors were found to contain rchd534 cDNAs. Plasmid tchv03A was

found to encode amino acids 17-235 of rchd534 and plasmid tchvR4A was found to encode amino acids 25-235 of rchd534.

It was confirmed that these rchd534 cDNAs encode proteins that physically interact specifically with fchd540. Yeast expression plasmids encoding the GAL4 DNA-binding domain either alone or fused in frame to fchd540, rchd534, Drosophila MAD, DPC4, and p53 were transformed into MAT α two-hybrid screening strain HF7c. Yeast expression plasmids encoding the GAL4 transcriptional activation domain (GAL4 AD) alone and GAL4 activation domain fusions to tchv03a, tchvR4A and SV40 were transformed into MAT α two-hybrid screening strain Y187. p53 and SV40 interact with each other and should not interact with the experimental proteins. The HF7c transformants were propagated as stripes on semi-solid synthetic complete medium lacking L-leucine. Both sets of stripes were replica plated in the form of a grid onto a single rich YPAD plate and the haploid strains of opposite mating types were allowed to mate overnight at 30°C. The yeast strains on the mating plate were then replica plated to a synthetic complete plate lacking L-leucine and L-tryptophan to select for diploids and incubated at 30°C overnight. Diploid strains on the synthetic complete plate lacking L-leucine and L-tryptophan were replica plated to a synthetic complete plate lacking L-leucine, L-tryptophan, and L-histidine to assay HIS3 expression and a paper filter on a synthetic complete plate lacking L-leucine and L-tryptophan. The next day the paper filter was subjected to the paper filter beta-galactosidase assay to measure expression of the lacZ reporter gene. HIS3 expression was scored after 3 days of growth at 30°C. The results are shown in the table below. The strength or absence of physical interaction between each combination of test proteins is listed. Strong interactions are defined as interactions that cause the activation of both the HIS3 and lacZ reporter genes.

35

TABLE 3

		cDNA-GAL4 Activation Domain Fusion Tested				
5		rchd534	tchv03A	tchvR4A	SV40	GAL4 AD alone
	<u>GAL4 DNA- Binding Domain Fusions</u>					
10	fchd540	Strong	Strong	Strong	None	None
	rchd534	None	None	None	None	None
	Dros. MAD	None	None	None	None	None
	DPC4	None	None	None	None	None
	p53	None	None	None	Strong	None
15	GAL4 DNA- Binding Domain alone	None	None	None	None	None

20 The tchv03A and tchvR4A fish proteins were found to interact strongly with the fchd540 bait protein and to not interact with the rchd534, MAD, DPC4, p53, and GAL4 DNA binding domain bait proteins. These results confirm the result that the rchd534 and fchd540 proteins interact strongly with each other.

25

11.3. Further Analysis of rchd534 and fchd540 Function

The significance of the rchd534/fchd540 protein interaction was confirmed by examination of their expression and activity in human cells and animal models.

30

11.3.1. Tissue Expression Patterns

35 The expression patterns were examined using in situ hybridization techniques. Fluorescently labeled DNA probes of both the rchd534 and fchd540 genes were used to probe human carotid endarterectomy samples. The expression of rchd534 and fchd540 was specific to endothelial cells lining the luminal surface of the carotid artery. Neither gene

showed expression in any other cell type present in the arterial tissue sample, including smooth muscle cells and macrophages. Thus, the specificity of their expression in a cell-type that is found only in vascular tissue, including atherosclerotic plaques, in addition to their up-regulation under the shear stress cardiovascular disease paradigm, indicate that rchd534 and fchd540 are excellent and specific targets for therapeutic intervention.

11.3.2. Cellular Localization

The cellular localization of the rchd534 and fchd540 proteins in bovine aortic endothelial cells (BAECs) was examined in relationship to other proteins involved in the TGF- β signalling pathway. In all experiments, the rchd534 and fchd540 proteins were located in the cytoplasm. MADR2 was located in the cytoplasm when transfected alone and in the nucleus when co-transfected with activated T β RI or when TGF- β was added to the culture medium. Co-transfection of rchd534 or fchd540 with MADR2 prevented the localization of MADR2 in the nucleus in response to TGF- β signalling.

11.3.3. Protein Interactions in Human Cells

The interaction of the rchd534 and fchd540 proteins, observed in yeast cells as described above, was tested in mammalian endothelial cell tissue culture. Either bovine aortic endothelial cells (BAECs) or 293 cells (human embryonic kidney cells, ATCC Accession No. CRL-1573) were transfected with constructs encoding both the rchd534 and fchd540 proteins, each fused to a different flag peptide allowing for specific immunoprecipitation. The rchd534 and fchd540 proteins were found to co-immunoprecipitate as heterodimers in extracts produced from both 293 cells and BAECs. The co-immunoprecipitation of rchd534 and fchd540 further supports that these proteins interact in human cells that are physiologically relevant to cardiovascular disease.

The ability of the rchd534 and fchd540 proteins to interact with themselves and with other protein members of the TGF- β

signalling pathway (MADR1, MADR2, DPC4, Tbr1, TSR1, ActR1b, ALK3, ALK6), was tested using this co-immunoprecipitation method. Each gene was transfected alone and in various combinations with other TGF- β pathway genes in either 293 cells or BAECs. The rchd534 protein formed homodimers in 293 cells and BAECs. The fchd540 protein did not form homodimers in 293 cells or BAECs. As mentioned above, the rchd534 and fchd540 proteins formed heterodimers in 293 cells and BAECs. This interaction is about 50 fold stronger in BAECs than 293 cells based on equal amounts of protein. However, the rchd534-fchd540 protein interaction was significantly less avid than the rchd534 protein's interaction with itself.

The rchd534 protein interacted with MADR1, MADR2, and DPC4 in 293 cells and BAECs. The strength of MADR1 and MADR2 interactions was about the same between 293 cells and BAECs and much greater in BAECs for DPC4. The fchd540 protein interacted very weakly with MADR1, MADR2, and DPC4 in 293 cells. The rchd534 protein interacted strongly with activated forms of T β RI and ActRI and weakly with activated ALK6 in 293 cells. The fchd540 protein interacted strongly with activated T β RI and ALK6 receptors, and weakly with activated forms of TSRI, ALK3, and ActRIb in 293 cells. Thus, in addition to the interaction of the rchd534 and fchd540 proteins, the interaction of the rchd534 protein with itself, as well as the interaction of the rchd534 protein and the fchd540 protein with the other proteins in the TGF- β pathway described above are excellent targets for therapeutic intervention.

11.3.4. Effect of Expression on TGF- β Signalling

The effect of both rchd534 and fchd540 on the TGF- β signalling pathway was tested in vitro. Primary BAECs were transfected with a construct called p3TP-Lux, containing a TGF- β responsive promoter fused to a reporter gene (Wrana et al., 19944, Nature 370: 341-347). The rchd534 gene or the fchd540 gene in pCI expression vectors (Promega) was transfected with and without MADR1 (pCMV5MADR1-Flag, Hoodless

et al. 1996 Cell 85: 489-500) or MADR2 (pCMV5MADR2-Flag, Eppert et al. 1996 Cell 86: 543-552). The TGF- β response was induced 20-fold by either MADR1 or MADR2. Co-expression of either rchd534 or fchd540 completely eliminated this
5 induction. Thus, the rchd534 and fchd540 proteins inhibited MADR1- and MADR2-mediated TGF- β signalling in endothelial cells. These results further demonstrate that the interactions of either the rchd534 protein or the fchd540 protein with MADR2 or with activated T β R1 are excellent
10 targets for therapeutic intervention. As described above, the expression of rchd534 and fchd540 is specific, within arterial tissue, to endothelial cells. Accordingly, the rchd534 and rchd540 genes may be targets for intervention in a variety of inflammatory and fibroproliferative disorders
15 that involve endothelial cells, including, but not limited to, cancer angiogenesis, inflammation, and fibrosis.

12. EXAMPLE: ANTISENSE AND RIBOZYME MOLECULES FOR
 INHIBITION OF RCHD534 AND FCHD540 EXPRESSION

20 The principles presented in Section 5.6.1.1, above, can be used to design oligonucleotides for use in inhibiting the expression of target genes, such as the rchd534 or fchd540 genes.

25 The following antisense molecules can be used to inhibit the expression of the rchd534 gene:

Antisense:

- 30 a) 5'-CATTTTCATTTTCATACAA-3' which is complementary to nucleotides -14 to +3 of rchd534 in FIG.6.
- b) 5'-CATTTTCATTTTCATACAATATATG-3' which is complementary to nucleotides -20 to +3 of rchd534 in FIG.6.
- 35 c) 5'-CATTTTCATTTTCATACAATATATGGCCTTT-3' which is complementary to nucleotides -26 to -3 of rchd534 in FIG.6.

- d) 5'-CATTTTCATTTTCATACAATATATGGCCTTTTGTGGC-3' which is complementary to nucleotides -32 to +3 of rchd534 in FIG.6.
- 5 e) 5'-GGACATTTTCATTTTCATACAATATATGGCCTTTTGT-3' which is complementary to nucleotides -29 to +6 of rchd534 in FIG.6.
- 10 f) 5'-TTCATTTTCATACAATATATGGCCTTTTGT-3' which is complementary to nucleotides -29 to -1 of rchd534 in FIG.6.
- g) 5'-TCATACAATATATGGCCTTTTGT-3' which is complementary to nucleotides -29 to -7 of rchd534 in FIG.6.
- 15 h) 5'-AATATATGGCCTTTTGT-3' which is complementary to nucleotides -29 to -13 of rchd534 in FIG.6.

The following antisense molecules can be used to inhibit the expression of the fchd540 gene:

- 20 a) 5'-CATGCGGGGCGAGGAGG-3' which is complementary to nucleotides -14 to +3 of fchd540 in FIG.2.
- b) 5'-CATGCGGGGCGAGGAGGCGAGGA-3' which is complementary to nucleotides -20 to +3 of fchd540 in FIG.2.
- 25 c) 5'-CATGCGGGGCGAGGAGGCGAGGAGAAAAG-3' which is complementary to nucleotides -26 to +3 of fchd540 in FIG.2.
- 30 d) 5'-CATGCGGGGCGAGGAGGCGAGGAGAAAAGTCGTTT-3' which is complementary to nucleotides -32 to +3 of fchd540 in FIG.2.
- 35 e) 5'-GAACATGCGGGGCGAGGAGGCGAGGAGAAAAGTCG-3' which is complementary to nucleotides -29 to +6 of fchd540 in FIG.2.

- f) 5'-GCGGGGCGAGGAGGCGAGGAGAAAAGTCG-3' which is complementary to nucleotides -29 to -1 of fchd540 in FIG.2.
- 5 g) 5'-CGAGGAGGCGAGGAGAAAAGTCG-3' which is complementary to nucleotides -29 to -7 of fchd540 in FIG.2.
- h) 5'-GGCGAGGAGAAAAGTCG-3' which is complementary to nucleotides -29 to -13 of fchd540 in FIG.2.

10

Ribozymes:

The central, catalytic portion of a hammerhead ribozyme molecule consist of the following sequence:

5'-CAAAGCNGNXXXXNCNGAGNAGUC-3';

wherein the 5'-proximal CA bases hybridize to a complementary 5'-UG-3' in the target mRNA. The first four underlined bases form a stem by base pairing with the second set of underlined bases, with the intervening bases, shown as X's, forming a non-pairing loop. In order to hybridize to a target mRNA, a hammerhead ribozyme contains additional bases flanking each end of the central segment shown above. The 5' ribozyme flanking segment is complementary to the respective flanking sequences immediately 3' to the target UG; and the 3' flanking segment is complementary to the respective flanking sequence beginning two bases upstream of the target U, and extending 5'-ward (in effect, skipping the first base upstream of the target U). Cleavage occurs between first and second bases upstream of (i.e., 5' to) the U in the target 5'-UG-3' site.

35

The following ribozyme molecules can be used to inhibit the expression of the rchd534 gene:

- a) 5'-GGUGGAGCCCCAGGGCAUUACCUCAAAGCNGNXXXXXNCNGAGNAGUCGUGG
5 GCAAGGUGGGCACUCAGGUGGG-3' which will cleave the
rchd534 mRNA between nucleotides 716 and 717 in FIG.6.
- b) 5'-GUGUCUCUAUGGGUUUGCCCAAAGCNGNXXXXXNCNGAGNAGUCUCUGGACA
UUUCAUUUCAUAC-3' which will cleave the rchd534 mRNA
between nucleotides 1040 and 1041 in FIG.6.
- 10 c) 5'-GGCCCUCUCGCCGUCGGGCUCCUUGCUGAGCAAAGCNGNXXXXXNCNGAGNA
GUCGAUGCCGAAGCCGAUCUUGCUGCGCG-3' which will cleave
between nucleotides 1421 and 1422 in FIG.6.

The following ribozyme molecules can be used to inhibit the expression of the fchd540 gene:

- a) 5'-CGUUUGCCUGCUAAGGAGCGAACAAAGCNGNXXXXXNCNGAGNAGUCGAUGU
UUCUUUGUGAGUCGGGCGCCG-3', which will cleave the
fchd540 mRNA between nucleotides -53 and -52 in FIG.2.
- 20 b) 5'-CGCCGGACGAGCGCAGAUUCGUUUGGUCCUGAACAAAGCNGNXXXXXNCNGAG
NAGUCCGGGGCGAGGAGGCGAGGAGAAAAGUCG-3', which will
cleave the fchd540 mRNA between nucleotides -1 and +1
in FIG.2.
- c) 5'-GGAGUAAGGAGGGGGGGGAGACUCUAGUUCGCAAAGCNGNXXXXXNCNGAGN
25 AGUCAGUCGGCUAAGGUGAUGGGGGUUGCAGCACACC-3' which will
cleave the fchd540 mRNA between nucleotides +602 and
+603 in FIG.2.

30

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13. DEPOSIT OF MICROORGANISMS

The following microorganisms were deposited with the American Type Culture Collection (ATCC), Rockville, Maryland, 5 on the indicated dates and assigned the indicated accession numbers:

	<u>Microorganism</u>	<u>ATCC Accession No.</u>	<u>Date of Deposit</u>
	pFCHD531	69983	February 7, 1996
10	pFCHD540	69984	February 7, 1996
	fchd545	69974	January 5, 1996

The following microorganism was deposited with the Agricultural Research Service Culture Collection (NRRL), 15 Peoria, Illinois, on the indicated date and assigned the indicated accession number:

	<u>Microorganism</u>	<u>NRRL Accession No.</u>	<u>Date of Deposit</u>
20	FCHD534	B-21459	June 6, 1995

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended as single illustrations of individual aspects of the 25 invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying 30 drawings. Such modifications are intended to fall within the scope of the appended claims.

International Application No: PCT/

MICROORGANISMS	
Optional Sheet in connection with the microorganism referred to on page <u>138</u> , lines <u>1-35</u> of the description *	
A. IDENTIFICATION OF DEPOSIT * Further deposits are identified on an additional sheet *	
Name of depositary institution * American Type Culture Collection	
Address of depositary institution (including postal code and country) * 12301 Parklawn Drive Rockville, MD 20852 US	
Date of deposit * February 7, 1996 Accession Number * 69983	
B. ADDITIONAL INDICATIONS * (leave blank if not applicable). This information is continued on a separate attached sheet	
C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE * (if the indications are not all designated States)	
D. SEPARATE FURNISHING OF INDICATIONS * (leave blank if not applicable) The indications listed below will be submitted to the International Bureau later * (Specify the general nature of the indications e.g., "Accession Number of Deposit")	
E. <input type="checkbox"/> This sheet was received with the International application when filed (to be checked by the receiving Office)	
<div style="text-align: right;">_____ (Authorized Officer)</div>	
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Form PCT/RO/134 (January 1981)

International Application No: PCT/ /

Form PCT/RO/134 (cont.)

American Type Culture Collection

12301 Parklawn Drive
Rockville, MD 20852
US

<u>Accession No.</u>	<u>Date of Deposit</u>
69984	February 7, 1996
69974	January 5, 1996

Agricultural Research Culture Collection (NRRL)

International Depository Authority
1815 N. University Street
Peoria, IL 61604
US

B-21459	June 6, 1995
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WHAT IS CLAIMED IS:

1. An isolated polynucleotide containing the following nucleotide sequence:
 - 5 fchd531 (SEQ ID NO.:1),
fchd540 (SEQ ID NO.:2),
fchd545 (SEQ ID NO.:3),
fchd602 (SEQ ID NO.:4) or,
fchd605 (SEQ ID NO.:5).
- 10 or the nucleotide sequence of a gene or gene fragment contained in the following clone as deposited with the ATCC:
pFCHD531 (in ATCC Accession No. 69983),
pFCHD540 (in ATCC Accession No. 69984), or
fchd545 (in ATCC Accession No. 69974).
- 15
2. An isolated polynucleotide which hybridizes under stringent conditions to the nucleotide sequence of Claim 1 or its complement, or to the gene or gene fragment contained in the clone of Claim 1 as deposited with the ATCC.
- 20
3. An isolated polynucleotide which encodes an amino acid sequence encoded by the nucleotide sequence of Claim 1 or its complement, or encoded by the gene or gene fragment contained in the clone of Claim 1 as deposited with the ATCC.
- 25
4. A polynucleotide vector containing the nucleotide sequence of Claim 1, 2 or 3.
5. A polynucleotide expression vector containing the
30 nucleotide sequence of Claim 1, 2 or 3 in operative association with a nucleotide regulatory element that controls expression of the nucleotide sequence in a host cell.
- 35
6. A genetically engineered host cell containing the polynucleotide of Claim 1, 2 or 3.

7. A genetically engineered host cell containing the polynucleotide of Claim 1, 2 or 3 in operative association with a nucleotide regulatory element that controls expression of the nucleotide sequence in the host cell.

5

8. A substantially pure gene product encoded by the polynucleotide of Claim 1, 2, or 3.

9. An antibody that immunospecifically binds the gene
10 product of Claim 8.

10. A transgenic animal in which the polynucleotide of Claim 1, 2 or 3 is an expressed transgene contained in the genome of the animal.

15

11. A transgenic animal in which expression of genomic sequences encoding the gene product of Claim 8 is prevented or suppressed.

20 12. A method for diagnosing cardiovascular disease, comprising assaying, in a patient sample, the differential expression of a gene encoding the fchd531 protein, the fchd540 protein, the fchd545 protein, the fchd602 protein, or the fchd605 protein.

25

13. The method of Claim 12 in which the cardiovascular disease is atherosclerosis.

14. The method of Claim 12 in which the cardiovascular
30 disease is ischemia/reperfusion.

15. The method of Claim 12 in which the cardiovascular disease is hypertension.

35 16. The method of Claim 12 in which the cardiovascular disease is restenosis.

17. The method of Claim 12 in which the gene is up-regulated.
18. The method of Claim 17 in which the gene encodes the 5 fchd540, fchd602, or fchd605 protein.
19. The method of Claim 12 in which the gene is down-regulated.
- 10 20. The method of Claim 19 in which the gene encodes the fchd531 or fchd545 protein.
21. A method for treating cardiovascular disease, comprising administering a compound that modulates the 15 expression of, or the activity of the encoded protein product of, the fchd531 gene, the fchd540 gene, the fchd545 gene, the fchd602 gene, or the fchd605 gene to a patient in need of such treatment.
- 20 22. The method of claim 21 in which the cardiovascular disease is atherosclerosis.
23. The method of claim 21 in which the cardiovascular disease is ischemia/reperfusion.
- 25 24. The method of claim 21 in which the cardiovascular disease is hypertension.
25. The method of claim 21 in which the cardiovascular 30 disease is restenosis.
26. The method of Claim 21 in which the compound inhibits the expression of the gene or the activity of the protein product.
- 35 27. The method of Claim 26 in which the gene is the fchd540, fchd602, or fchd605 gene.

28. The method of Claim 27 in which the compound is an antisense or ribozyme molecule that blocks translation of the gene.

5 29. The method of Claim 27 in which the compound is complementary to the 5' region of the gene and blocks transcription via triple helix formation.

30. The method of Claim 26 in which the compound is an
10 antibody that inhibits the activity of the protein product.

31. The method of Claim 21 in which the compound enhances the expression of the gene or the activity the protein product.

15

32. The method of Claim 31 in which the gene is the fchd531 or fchd545 gene.

33. A method for treating cardiovascular disease,
20 comprising administering a polynucleotide encoding the fchd531 or fchd545 protein to a patient in need of such treatment.

34. A method for treating cardiovascular disease,
25 comprising administering an effective amount of the fchd531 or fchd545 protein to a patient in need of such therapy.

35. A method for monitoring the efficacy of a compound in clinical trials for the treatment of cardiovascular disease,
30 comprising assaying, in a patient sample, the differential expression of a gene encoding the fchd531 protein, the fchd540 protein, the fchd545 protein, the fchd602 protein, or the fchd605 protein.

35 36. The method of Claim 35 in which the cardiovascular disease is atherosclerosis.

37. The method of Claim 35 in which the cardiovascular disease is ischemia/reperfusion.

38. The method of Claim 35 in which the cardiovascular disease is hypertension.

39. The method of Claim 35 in which the cardiovascular disease is restenosis.

40. The method of Claim 35 in which the gene is up-regulated.

41. The method of Claim 40 in which the gene encodes the fchd540, fchd602, or fchd605 protein.

15

42. The method of Claim 35 in which the gene is down-regulated.

43. The method of Claim 42 in which the gene encodes the fchd531 or fchd545 protein.

44. A method for identifying a substance for treating cardiovascular disease comprising assaying the ability of the substance to modulate the expression of, or the activity of the encoded protein product of, the fchd531 gene, the fchd540 gene, the fchd545 gene, the fchd602 gene, or the fchd605 gene.

45. The method of Claim 44 in which the cardiovascular disease is atherosclerosis.

46. The method of Claim 44 in which the cardiovascular disease is ischemia/reperfusion.

47. The method of Claim 44 in which the cardiovascular disease is hypertension.

48. The method of Claim 44 in which the cardiovascular disease is restenosis.

49. The method of Claim 44 in which the modulation of expression of said gene is assayed by:

- (a) exposing a sample of cells to a test substance;
- (b) assaying the expression of said gene in the sample of cells; and
- (c) comparing the expression level of the gene in the sample exposed to the substance to the expression level of the gene in a control sample of cells, in which a difference between the expression level of the gene in the sample exposed to the substance and the control indicates the modulation of expression of the gene.

15

50. The method of Claim 49 in which the gene is down-regulated by the test substance.

51. The method of Claim 50 in which the substance is an oligonucleotide complementary to the 5' region of the gene and blocks transcription via triple helix formation.

52. The method of Claim 50 in which the substance is an antisense or ribozyme molecule that blocks translation of the gene.

53. The method of Claim 49 in which the gene is up-regulated by the test substance.

54. The method of claim 44 in which the substance is a small organic or inorganic molecule that modulates the activity of the protein product by binding to the protein product.

55. The method of claim 44 in which the substance is an antibody that modulates the activity of the protein product by binding to the protein product.

56. An assay for identifying a substance that binds to the fchd531 protein, the fchd540 protein, the fchd545 protein, the fchd602 protein, or the fchd605 protein, comprising:

- (a) contacting a protein or peptide containing an amino acid sequence corresponding to the binding site of the protein with a test substance, under conditions and for a time sufficient to permit binding and formation of a complex between the protein or peptide and the test substance, and
- (b) detecting the formation of a complex, in which the ability of the test substance to bind to the protein is indicated by the presence of the test substance in the complex.

57. An assay for identifying a substance that inhibits the interaction between the rchd534 protein and the fchd540 protein comprising:

- (a) contacting a protein or peptide containing an amino acid sequence corresponding to the binding site of the rchd534 protein with a protein or peptide containing an amino acid sequence corresponding to the binding site of the fchd540 protein, under conditions and for a time sufficient to permit binding and formation of a complex, in the presence of a test substance, and
- (b) detecting the formation of a complex, in which the ability of a test substance to inhibit the interaction between the rchd534 protein and fchd540 protein is indicated by a decrease in complex formation as compared to the amount of complex formed in the absence of the test substance.

58. An assay for identifying a substance that inhibits the interaction between two rchd534 protein molecules comprising:

- (a) contacting a first protein or peptide containing an amino acid sequence corresponding to the binding site of the rchd534 protein with a second protein or peptide containing an amino acid sequence corresponding to the binding site of the rchd534 protein, under conditions and for

a time sufficient to permit binding and formation of a complex, in the presence of a test substance, and

- (b) detecting the formation of a complex, in which the ability of a test substance to inhibit the interaction between two rchd534 protein molecules is indicated by a decrease in complex formation as compared to the amount of complex formed in the absence of the test substance.

59. An assay for identifying a substance that inhibits the interaction between the rchd534 protein and a protein member of the TGF- β signalling pathway comprising:

- (a) contacting a protein or peptide containing an amino acid sequence corresponding to the binding site of the rchd534 protein with a protein or peptide containing an amino acid sequence corresponding to the binding site of the protein member of the TGF- β signalling pathway, under conditions and for a time sufficient to permit binding and formation of a complex, in the presence of a test substance, and
- (b) detecting the formation of a complex, in which the ability of a test substance to inhibit the interaction between the rchd534 protein and the protein member of the TGF- β signalling pathway is indicated by a decrease in complex formation as compared to the amount of complex formed in the absence of the test substance.

60. The assay of Claim 59 wherein the protein member of the TGF- β signalling pathway is MADR1, MADR2, DPC4, activated T β R1, activated ActR1b, or activated ALK6.

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61. An assay for identifying a substance that inhibits the interaction between the fchd540 protein and a protein member of the TGF- β signalling pathway comprising:

- (a) contacting a protein or peptide containing an amino acid sequence corresponding to the binding site of the fchd540 protein with a protein or peptide containing an amino acid sequence corresponding to the binding site of the

protein member of the TGF- β signalling pathway, under conditions and for a time sufficient to permit binding and formation of a complex, in the presence of a test substance, and

- 5 (b) detecting the formation of a complex, in which the ability of the test substance to inhibit the interaction between the fchd540 protein and the protein member of the TGF- β signalling pathway is indicated by a decrease in complex formation as compared to the amount of complex formed
10 in the absence of the test substance.

62. The assay of Claim 61 wherein the protein member of the TGF- β signalling pathway is MADR1, MADR2, DPC4, activated T β R1, activated ALK6, activated TSR1, activated ALK3, or
15 activated ActR1 β .

63. A method for treating cardiovascular disease comprising administering a compound that inhibits the interaction between the rchd534 protein and the fchd540
20 protein.

64. A method for treating cardiovascular disease comprising administering a compound that inhibits the interaction between two rchd534 protein molecules.
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65. A method for treating cardiovascular disease comprising administering a compound that inhibits the interaction between the rchd534 protein and a protein member of the TGF- β signalling pathway.
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66. The method of Claim 65 wherein the protein member of the TGF- β signalling pathway is MADR1, MADR2, DPC4, activated T β R1, activated ActR1b, or activated ALK6.

35 67. A method for treating cardiovascular disease comprising administering a compound that inhibits the

interaction between the fchd540 protein and a protein member of the TGF- β signalling pathway.

68. The method of Claim 67 wherein the protein member of the TGF- β signalling pathway is MADR1, MADR2, DPC4, activated T β R1, activated ALK6, activated TSR1, activated ALK3, or activated ActR1 β .

69. A method for identifying a substance that enhances the TGF- β signalling response comprising:

(a) contacting a genetically engineered cell with a test substance, said cell comprising 1) a reporter gene in operative association with an inducible TGF- β regulatory element; 2) a recombinant gene encoding the rchd534 protein or a recombinant gene encoding the fchd540 protein; and 3) a recombinant gene encoding the MADR1 protein or a recombinant gene encoding the MADR2 protein; and

(b) detecting expression of said reporter gene in which ability of the test substance to enhance the TGF- β signalling response is indicated by an increase in expression of the reporter gene as compared to the amount of expression in the absence of the test substance.

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GGCACGAGTCGGAGCCGGG

CGGAGGGGAGGGGGAAAGAGGAGCGCAGGGTGAGAGTGAGCCGACGGCTTCGGGAGGCGAGGGGGCGGGGGAGCAGC

	M	P	G	E	
GCCGAGGYCGCCGCTCCGCCCTCCGGCCCTAGGACTAGGGGTGGGGACGGACAAGCCCCCG	ATG	CCG	GGG	GAG	4 12
T E E P R P P E Q Q Q D Q E G G E A A K A					24
ACG GAA GAG CCG AGA CCC CCG GAG CAG CAG GAC GAG GAA GGG GGA GAG GCG GCC AAG GCG					72
A P E E P Q Q R P P E A V A A A P A G T					44
GCT CCG GAG GAG CCC CAA CAG CCC CCT GAG GCG GTC GCG GCG CCT GCA GGG ACC					132
T S S R V L R G G R D R G R A A A A A					64
ACT AGC AGC CGC GTG CTG AGG GGA GGT CGG GAC CGA GGC CGG GCC GCT GCG GCC GCC GCC					192
A A A V S R R R K A E Y P R R R R S S P					84
GCC GCA GCT GTG TCC CGC CGG AGG AAG GCC GAG TAT CCC CGC CGG CGG AGG AGC AGC CCC					252
S A R P P D V P G Q Q P Q A A K S P S P					104
AGC GCC AGG CCT CCC GAC GTC CCC GGG CAG CCC CAG GCC GCG AAG TCC CCG TCT CCA					312
V Q G K K S P R L L C I E K V T T D K D					124
GTT CAG GGC AAG AAG AGT CCG CGA CTC CTA TGC ATA GAA AAA GTA ACA ACT GAT AAA GAT					372
P K E E K E E D D S A L P Q E V S I A					144
CCC AAG GAA GAA AAA GAG GAA GAA GAC GAT TCT GCC CTC CCT CAG GAA GTT TCC ATT GCT					432

FIG.1A

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A S R P S R G W R S S R T S V S R H R D 164
 GCA TCT AGA CCT AGC CGG GGC TGG CGT AGT AGT AGG ACA TCT GTT TCT CGC CAT CGT GAT 492

 T E N T R S S R S K T G S L Q L I C K S 184
 ACA GAG AAC ACC CGA AGC TCT CGG TCC AAG ACC GGT TCA TTG CAG CTC ATT TGC AAG TCA 552

 E P N T D Q L D Y D V G E E H Q S P G G 204
 GAA CCA AAT ACA GAC CAA CTT GAT TAT GAT GTT GGA GAA GAG CAT CAG TCT CCA GGT GGC 612

 I S G E E E E E E E M L I S E E E I 224
 ATT AGT GGT GAA GAG GAA GAG GAG GAA GAG ATG TTA ATC AGT GAA GAG GAG ATA 672

 P F K D D P R D E T Y K P H L E R E T P 244
 CCA TTC AAA GAT GAT CCA AGA GAT GAG ACC TAC AAA CCC CAC TTA GAA AGG GAA ACC CCA 732

 K P R R K S G K V K E E K E K E I K V 264
 AAG CCA CGG AGA AAA TCA GGG AAG GTA AAA GAA GAG AAG GAG AAG AAG GAA ATT AAA GTG 792

 E V E V E V K E E E N E I R E D E E P P 284
 GAA GTA GAG GTG GAG GTG AAA GAA GAG GAG AAT GAA ATT AGA GAG GAT GAG GAA CCT CCA 852

 R K R G R R R K D D K S P R L P K R R K 304
 AGG AAG AGA GGA AGA AGA CGA AAA GAT GAC AAA AGT CCA CGT TTA CCC AAA AGG AGA AAA 912

 K P P I Q Y V R C E M E G C G T V L A H 324
 AAG CCT CCA ATC CAG TAT GTC CGI TGT GAG ATG GAA GGA TGT GGA ACT GTC CTT GCC CAT 972

FIG.1B

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P R Y L Q H H I K Y Q H L L K K K Y V C 344
 CCT CGC TAT TTG CAG CAC CAC ATT AAA TAC CAG CAT TTG CTG AAG AAG AAA TAT GTA TGT 1032

 P H P S C G R L F R L Q K Q L L R H A K 364
 CCC CAT CCC TCC TGT GGA CGA CTC TTC AGG CTT CAG AAG CAA CTT CTG CGA CAT GCC AAA 1092

 H H T D Q R D Y I C E Y C A R A F K S S 384
 CAT CAT ACA GAT CAA AGG GAT TAT ATC TGT GAA TAT TGT GCT CGG GCC TTC AAG AGT TCC 1152

 H N L A V H R M I H T G E K P L Q C E I 404
 CAC AAT CTG GCA GTG CAC CGG ATG ATT CAC ACT GGC GAG AAG CCA TTA CAA TGT GAG ATC 1212

 C G F T C R Q K A S L N W H M K K H D A 424
 TGT GGA TTT ACT TGT CGA CAA AAG GCA TCT CTT AAT TGG CAC ATG AAG AAA CAT GAT GCA 1272

 D S F Y Q F S C N I C G K K F E K K D S 444
 GAC TCC TTC TAC CAG TTT TCT TGC AAT ATC TGT GGC AAA AAA TTT GAG AAG AAG GAC AGC 1332

 V V A H K A K S H P E V L I A E A L A A 464
 GTA GTG GCA CAC AAG GCA AAA AGC CAC CCT GAG GTG CTG ATT GCA GAA GCT CTG GCT GCC 1392

 N A G A L I T S T D I L G T N P E S L T 484
 AAT GCA GGC GCC CTC ATC ACC AGC ACA GAT ATC TTG GGC ACT AAC CCA GAG TCC CTG ACG 1452

 Q P S D G Q G L P L L P E P L G N S T S 504
 CAG CCT TCA GAT GGT CAG GGT CTT CCT CTT CTT GAG CCC TTG GGA AAC TCA ACC TCT 1512

FIG.1C

FIG. 1D

GCACGAGCGGAGAGCCGGCGCAGGGCGCGGGCCGCGGGGTGGGGCAGCCGGAGCGCA
GGCCCCCGATCCCCGGGGGGCCCCCGGGGCCCCCGGGCGGCCCCCGGCCCTCCGGGAGACTGGCGCATGCCACGGAGCG
CCCCTGGGGCGCGCGCTCCTGCCCCGGGCCCCCTGCTGCTGCTGCTGCGCCTGCGCCTGCTGCCCAACTCGGGCGCC
CGACTCACAAAGAACATCATGTTGCTCCTTAGCAGGCAACGACTTTTCTCCTCGCCTCCTCGCCCCGC ATG TTC
M F 2 6
R T K R S A L V R R L W R S R A P G G E 22
AGG ACC AAA CGA TCT GCG CTC GTC CGG CGT CTC TGG AGG AGC CGT GCG CCC GGC GGC GAG 66
D E E E G A G G G G G G G G E L R G E G A 42
GAC GAG GAG GAG GCA GGG GGA GGT GGA GGA GGC GAG CTG CCG GGA GAA GGC GCG 126
T D S R A H G A G G G G G G G G P G R A G C C L 62
ACG GAC AGC CGA GCG CAT GGG GCC GGT GGC GGC GGC CCG GGC AGG GCT GGA TGC TGC CTG 186
G K A V R G A K G H H H P H P A A G A 82
GGC AAG GCG GTG CGA GGT GCC AAA GGT CAC CAC CAT CCC CAC CCG CCA GCC GCG GGC GCC 246
G A A G G A E A D L K A L T H S V L K K 102
GGC GCG GCC GGG GGC GCC GAG GCG GAT CTG AAG GCG CTC ACG CAC TCG GTG CTC AAG AAA 306
L K E R Q L E L L L Q A V E S R G G T R 122
CTG AAG GAG CGG CAG CTG GAG CTG CTC CAG GCC GTG GAG TCC CGC GGC GGC ACG CGC 366

FIG.2A

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T A C L L L P G R L D C R L G L G P G A P A 142
 ACC GCG TGC CTC CTG CTG CCC GGC CGC CTG GAC TGC AGG CTG GGC CCG GGG GCG CCC GCC 426

 G A Q P A Q P P S S Y S L P L L L C K V 162
 GGC GCG CAG CCT GCG CAG CCG CCC TCG TCC TAC TCG CTC CCC CTC CTG TGC AAA GTG 486

 F R W P D L R H S S E V K R L C C E S 182
 TTC AGG TGG CCG GAT CTC AGG CAT TCC TCG GAA GTC AAG AGG CTG TGT TGT GAA TCT 546

 Y G K I N P E L V C C N P H L S R L C 202
 TAC GGG AAG ATC AAC CCC GAG CTG GTG TGC TGC AAC CCC CAT CAC CTT AGC CGA CTC TGC 606

 E L E S P P P Y S R Y P M D F L K P T 222
 GAA CTA GAG TCT CCC CCT CCT TAC TCC AGA TAC CCG ATG GAT TTT CTC AAA CCA ACT 666

 A D C P D A V P S S A E T G G T N Y L A 242
 GCA GAC TGT CCA GAT GCT GTG CCT TCC TCC GCT GAA ACA GGG GGA ACG AAT TAT CTG GCC 726

 P G G L S D S Q L L L E P G D R S H W C 262
 CCT GGG GGG CTT TCA GAT TCC CAA CTT CTT CTG GAG CCT GGG GAT CGG TCA CAC TGG TGC 786

 V V A Y W E E K T R V G R L Y C V Q E P 282
 GTG GTG GCA TAC TGG GAG GAG AAG ACG AGA GTG GGG AGG CTC TAC TGT GTC CAG GAG CCC 846

 S L D I F Y D L P Q G N G F C L G Q L N 302
 TCT CTG GAT ATC TTC TAT GAT CTA CCT CAG GGG AAT GGC TTT TGC CTC GGA CAG CTC AAT 906

FIG.2B

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S D N K S Q L V Q K V R S K I G C G I Q 322
 TCG GAC AAC AAG AGT CAG CTG GTG CAG AAG GTG CGG AGC AAA ATC GGC TGC GGC ATC CAG 966

 L T R E V D G V W V Y N R S S Y P I F I 342
 CTG ACG CGG GAG GTG GAT GGT GTG TGG GTG TAC AAC CGC AGC AGT TAC CCC ATC TTC ATC 1026

 K S A T L D N P D S R T L L V H K V F P 362
 AAG TCC GCC ACA CTG GAC AAC CCT GAC TCC AGG ACG CTG TTG GTA CAC AAG GTG TTC CCC 1086

 G F S I K A F D Y E K A Y S L Q R P N D 382
 GGT TTC TCC ATC AAG GCT TTC GAC TAC GAG AAG GCG TAC AGC CTG CAG CGG CCC AAT GAC 1146

 H E F M Q Q P W T G F T V Q I S F V K G 402
 CAC GAG TTT ATG CAG CAG CCG TGG ACG GGC TTT ACC GTG CAG ATC AGC TTT GTG AAG GGC 1206

 W G Q C Y T R Q F I - S S C P C W L E V I 422
 TGG GGT CAG TGC TAC ACC CGC CAG TTC ATC AGC AGC TGC CCG TGC TGG CTA GAG GTC ATC 1266

 F N S R * 426
 TTC AAC AGC CGG TAG CCGCGTGGGAGGGGACAGAGCGTGAGCTGAGCAGGCCACACTTCAAACACTTTGCT 1278

 GCTAATATTTCCCTCCTGAGTGCCTTTCATGCAAACTCTTTGGCTTTTTTTTTTGTGTTGGTTGTTTCT
 TCTTCTCGTCTGTTTGTCIGTIIIGTTCGCCTTTGAGAAATAGCTTATGAAAAGAAATGTGGGGGTTTTT
 TGGAGAAGGGGCAGGTATGATCGGCAGGACACCCCTGATAGGAAGAGGGGAAGCAGAAATCCAAGCACCACCAACACA

FIG.2C

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GTGTATGAAGGGGGGGTTCATCTTCACTTGTCAAGAGTGTGTGTGAGTGTGAGTGTGCGGCTGTGTGTGCACGGCT
GTGCAGGAGCGGCAGATGGGGAGACAACGTGCTCTTTGTTTTGTGTCTTATGGATGTCCCCAGCAGAGAGGTTTGCA
GTCCCAAGCGGTGTCTCTCTGCCCTTGGACACGCTCAGTGGGGCAGAGGCAGTACCTGGGCAAGCTGGCGGCTGGGG
TCCCAGCAGCTGCCAGGAGCAGGCTCTGTCCCAGCCTGGGAAAGCCCTGCCCTCCTCTCCCTCATCAAGGACACG
GGCCTGTCCACAGGCTTCTGAGCAGCGAGCCTGCTAGTGGCCGAACCCAGAACCAATTAATTTTCATCCTTGTCTTATTC
CTTCTGCCAGCCCCCTGCCATTGTAGCGTCTTCTTTTTTGGCCAICGTCTCCTGGATCTCCCTGAGATGGGCTTCCCA
AGGGTGCCGGGGCAGCCCCCTCACAGTATTGCTACCCAGTGCCCTCTCCCCCTCAGCCTCTCCCCCTGCCCTGGCTGGT
GACATCAGGTTTTTCCCGGACTTAGAAAAACAGCTCAGCACTGCCCTGCTCCCATCCTGTGTGTTAAGCTCTGCTATTAG
GCCAGCAAGCGGGGATGTCCCTGGGAGGGACATGCTTAGCAGTCCCCCTTCCCTCCAAGAAGGATTTGGTCCGTCATAAC
CCAAGGTACCATCCTAGGCTGACACCTAACCTCTTCTTTCATTTCTTCTACAACTCATACACTCGTAIGATACTTCGACA
CTGTTCTTAGCTCAATGAGCATGTTTAGACTTTAACATAAGCTATTTTCTAACTACAAAGGTTTAAATGAACAAGAGA
AGCATCTCATTTGGAAATTTAGCATTTAGTGCTTTGAGAGAGAAAGGACTCCTGAAAAAAACCTGAGATTTATTAAA
GAAAAAATGTATTTATGTTATATATAAATATATTACTTGTAAATATAAAGACGTTTTTATAGCATCATTATTTA

FIG.2D

FIG. 2E

GGCACGAGGTTGCCCTGGCGGAGCAGAGACAGGCCCTCGGGTGGAGGTC

TTTGGTTTCATAAGAGCCTGAGAGAGATTTTCTAAGAT ATG TGT AAC ACA CCA ACG TAC TGT GAC CTA 10 30

G K A A K D V F N K G Y G F G M V K I D 30

GGA AAG GCT GCT AAG GAT GTC TTC AAC AAA GGA TAT GGC TTT GGC ATG GTC AAG ATA GAC 90

L K T K S C S G V E F S T S G H A Y T D 50

CTG AAA ACC AAG TCT TGT AGT GGA GTG GAA TTT TCT ACT TCT GGT CAT GCT TAC ACT GAT 150

T G K A S G N L E T K Y K V C N Y G L T 70

ACA GGG AAA GCA TCA GGC AAC CTA GAA ACC AAA TAT AAG GTC TGT AAC TAT GGA CTT ACC 210

F T Q K W N T D N T L G T E I S W E N K 90

TTC ACC CAG AAA TGG AAC ACA GAC AAT ACT CTA GGG ACA GAA ATC TCT TGG GAG AAT AAG 270

L A E G L K L T L D T I F V P N T G K K 110

TTG GCT GAA GGG TTG AAA CTG ACT CTT GAT ACC ATA TTT GTA CCG AAC ACA GGA AAG AAG 330

S G K L K A S Y K R D C F S V G S N V D 130

AGT GGG AAA TTG AAG GCC TCC TAT AAA CGG GAT TGT TTT AGT GTT GGC AGT AAT GTT GAT 390

I D F S G P T I Y G W A V L A F E G W L 150

ATA GAT TTT TCT GGA CCA ACC ATC TAT GGC TGG GCT GTG TTG GCC TTC GAA GGG TGG CTT 450

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FIG.3A

SUBSTITUTE SHEET (RULE 26)

FIG. 3B

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ACAGTGTAGCGTCATGTTAGAGGAGACGATCTGACCCACCAGTTTGTACATCAGTCCCTGTCATGTCCTCCACACCATTTTT
TCATGACCTTGTAATACTACTGGTCTCTGTGCTATAGTGAATCTTTGGTTTTGTCATCATAGTAAAAATAAAATAAACCCCA
TCACATTTGGAACATAAAAAAAAAAAAAAAAAA

FIG.3C

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T S L A L V L L N L L Q I Q R N V T L F P 20
 ACG AGC CTA GCC CTG GTG CTC AAC CTG CAG ATC CAG AGG AAT GTC ACT CTC TTC CCC 60

 E E V I A T I F S S A W V P C C G T 40
 GAG GAG GTG ATC GCC ACC ATC TTT TCC TCC GCC TGG TGG GTC CCT CCC TGC TGC GGG ACA 120

 A A V V G L L Y P C I D S H L G E P H 60
 GCA GCT GCT GTT GTT GGC CTA CTG TAC CCC TGT ATC GAC AGT CAC CTC GGA GAA CCC CAC 180

 K F K R E W A S V M R C I A V F V G I N 80
 AAA TTT AAG AGA GAA TGG GCC AGT GTC ATG CGC TGC ATA GCA GTT TTT GTT GGC ATT AAC 240

 H A S A K L D F A N N V Q L S L T L A A 100
 CAC GCC AGT GCT AAA TTG GAT TTT GCC AAT AAT GTC CAG CTG TCC TTG ACT TTA GCA GCC 300

 L S L G L W W T F D R S R S G L G L G I 120
 CTA TCT TTG GGC CTT TGG TGG ACA ITT GAT CGT TCC AGA AGT GGC CTT GGG CTG GGG ATC 360

 T I A F L A T L I T Q F L V Y N G V Y Q 140
 ACC ATA GCT TTT CTA GCT ACG CTG ATC ACG CAG TTT CTC GTG TAT AAT GGT GTC TAT CAG 420

 Y T S P D F L Y I R S W L P C I F F S G 160
 TAT ACA TCC CCA GAT TTC CTC TAT ATT CGT TCT TGG CTC CCT TGT ATA TTT TTC TCA GGA 480

 G V T V G N I G R Q L A M G V P E K P H 180
 GGC GTC ACG GTG GGG AAC ATA GGA CGA CAG TTA GCT ATG GGT GTT CCT GAA AAG CCC CAT 540

FIG.4A

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S D * 182
AGT GAT TGA GTCTTCAAAACCACCGATTCTGAGAGCAAGGAAGATTTTGGAGAGAAAATCTGACTGTGGATTATGAC 546
AAAGATTATCTTTTCTTAAGTAATCTATTTAGATCGGGCTGACTGTACAAATGACTCCTGGAAAAAACTCTTCACCT
AGCTAGAAATAGGGAGGTGGAGAAATGATGACTTACCCCTGAAGTCTTCCCTTGACTGCCCGCACTGGCGCCTGTCTGTGC
CCTGGAGCATTCTGCCCAGGCTACGTGGGTTCAGGCAGGTCAGAGCTTCCCAAGTATTCGATTTTCATGTGATTAA
AACAAGTTGCCATATTTCAAAAAAAAAAAAAAAAAMCTCGAGACCACCCCGCAGTTTGTGTGAGTCCCAAGGAGGT
AGGTTGATGGTGCTTAACAAACATGAAGTATGGTGTAAATAGGAATAATATTTATCCNAAAGATTTTAAAAAATAGGGCT
GTGTTTAAAAAAAAAAAAAAAAAAAA

FIG.4B

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M C H S R S C H P T M T I L Q A P T P A 20
 ATG TGT CAC TCT CGC AGC TGC CAC CCG ACC ATG ACC ATC CTG CAG GCC CCG ACC CCG GCC 60

 P S T I P G P R R G S G P E I F T F D P 40
 CCC TCC ACC ATC CCG GGA CCC CCG CGG GGC TCC GGT CCT GAG ATC TTC ACC TTC GAC CCT 120

 L P E P A A A P A G R P S A S R G H R K 60
 CTC CCG GAG CCC GCA GCG GCC CCT GCC GGG CGC CCC AGC GCC TCT CGC GGG CAC CGA AAG 180

 R S R R V L Y P R V V R R Q L P V E E P 80
 CGC AGC CGC AGG GTT CTC TAC CCT CGA GTG GTC CGG CGC CAG CTG CCA GTC GAG GAA CCG 240

 N P A K R L L F L L L T I V F C Q I L M 100
 AAC CCA GCC AAA AGG CTT CTC TTT CTG CTG CTC ACC ATC GTC TTC TGC CAG ATC CTG ATG 300

 A E E G V P A P L P P E D A P N A A S L 120
 GCT GAA GAG GGT GTG CCG GCG CCC CTG CCT CCA GAG GAC GCC CCT AAC GCC GCA TCC CTG 360

 A P T P V S P V L E P F N L T S E P S D 140
 GCG CCC ACC CCT GTG TCC CCC GTC CTC GAG CCC TTT AAT CTG ACT TCG GAG CCC TCG GAC 420

 Y A L D L S T F L L Q Q H P A A F * 157
 TAC GCT CTG GAC CTC AGC ACT TTC CTC CAG CAA CAC CCG GCC GCC TTC TAA 471

 CTGTGACTCCCCGCACTCCCCAAAAGAATCCGAAAAAACCAAAAGAAACACCCAGGCGTACCTGGTGGCGGAGAGCGTA 550

FIG. 5A

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TCCCCAACTGGGACTTCGGAGGCAACTTGAACTCAGAACTACAGCGGAGACGCCACCCGGTGCTTGAGGCGGGACCG 629

AGGCGCACAGAGACCGAGGGCGCATAGAGACCGAGGCACAGCCAGCTGGGGCTAGGCCCGGTGGGAAGGAGAGCGTCGT 708

TAATTTATTTCTTATTGCTCCTAAATAATTAATATGATTTATGTACGTCCTCCTAGGTGATGGAGATGIGTACGTA 787

ATATTTATTTTAACTTATGCAAGGGTGTGAGATGTTCCCTCTGCTGTAAATGCAGGTCTCTTGGTATTTTATGAGCTTT 866

GTGGGACTGGTGGAAAGCAGGACACCTTGGAACTGCGGCAAGTAGGAGAAATGGGGAGGACTCGGGTGGGGGAGGAC 945

GTCCCGGCTGGGATGAAGTCTGGTGGTGGTGGTAAAGTTTAGGAGGTGACTGCAATCCTCCAGCATCTCAACTCCGTCGTG 1024

TCTACTGTGTGAGACTTCGGCGGACCATTAGGAATGAGATCCGTGAGATCCTTCCATCTTCTTGAAGTCGCCCTTTAGGG 1103

TGGCTGCGAGGTAGAGGGTTGGGGGTTGGTGGGCTGTACGGAGCGACTGTCGAGATCGCCTAGTATGTTCTGTGAACA 1182

CAAAATAAAATTGATTTACTGTCAAAAAAAAAAAAAAAAAAACTCGAG 1228

FIG.5B

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GAATTCGGCACGAGGMCAGGAGCTCCTTTWCTGCGTCTCCCATCATGGGGCTTAGGGTTGAGTCTTCA 68
GGTTCGGGGGCAGGAAGGACGGGCACCTCAGGAGGCCCTCCCCATCCACAGCCCCCTCTTTGGGAGGGGGAAACTTIG 147
GCAACCCGGGAGGCATGTGGAICTTTTCCTAAGCAAGATGCTGAGCTGGAAGAATGGGGGTGTAAGGTAATGTCTCCCAA 226
CTGAAACTTTGCCAGGCACCTGGGAGAGGCTGTGAACTCTTTTCTGGCTTTAGAAATTTAGGTCTAGATCCCCAAAAGGCTA 305
AGTACCCCTGGGGGCTAACCAGAGGCATGCCCTGGGCTGAGCTGAACCTTCTGGTGCACTGGCCCCCTGGCTGACTGCTC 384
TTCTGCAGGAAGTTGGAGGAGATTCTTGAAGTTGATTCTCAGGCTGGATGTCCAAAGGGGTTGGAGTTTCTGATGTCT 463
TTCTGTCTCCCTCTCTTTTCTCTCCCTACCAGGTCCACTTCTTTCAGAGGGGCTGCGGTGCTCTAAAAGTTCTC 542
CTGTTAAAGTTTAGAGCAAAATGGTTATTATTTTAAATCAATAAAACTTTTAAAGTACTAAGACAACTTCTAAGAGG 621
GGAGTGGACAGAGGGCCTGGTGGCAGCTCACAGTTCTTTTCTGACCTTTGGTCTCACCCACCAAGTGTCACCTGAG 700
TGCCACCTTGCCCACTGAGGTAA TGCCCTGGGGCTCCACCAGTCCAGATCCACAGGGGCGCAGCCATGTGGGAGTGCC 779
GGCTGATTGTTACCCAGTAGTTGATAGCACATTATTATAACAGCCAAAGAGAGGAAGCAACCCCAAATGTCCATTAG 858
CTGATAAATGGATAAATGAAATATGGTACGTCCGAAGAATGGAATATCATTCACCCCTGAAAAAAGAACGAAAGTCCAGCA 937
CCAAAACGTGCTACAACA TGGATGAACCTTCGATGACTTTGTGCCACATGAAAGAAGAAGCCAGCCACAAAAAGGCCATAT 1016

FIG. 6A

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M S R M G K P I E T Q K S P P P 16
 ATTGTATGAAATGAA ATG TCC AGA ATG GGC AAA CCC ATA GAG ACA CAA AAA TCT CCG CCA CCT 1079

 P Y S R L S P R D E Y K P L D L S D S T 36
 CCC TAC TCT CGG CTG TCT CCT CGC GAC GAG TAC AAG CCA CTG GAT TCC GAT TCC ACA 1139

 L S Y T E T E A T N S L I T A P G E F S 56
 TTG TCT TAC ACT GAA ACG GAG GCT ACC AAC TCC CTC ATC ACT GCT CCG GGT GAA TTC TCA 1199

 D A S M S P D A T K P S H W C S V A Y W 76
 GAC GCC AGC ATG TCT CCG GAC GCC ACC AAG CCG AGC CAC TGG TGC AGC GTG GCG TAC TGG 1259

 E H R T R V G R L Y A V Y D Q A V S I F 96
 GAG CAC CGG ACG CGC GTG GGC CGC CTC TAT GCG GTG TAC GAC CAG GCC GTC AGC ATC TTC 1319

 Y D L P Q G S G F C L G Q L N L E Q R S 116
 TAC GAC CTA CCT CAG GGC AGC GGC TTC TGC TGC GGC CAG CTC AAC CTG GAG CAG CGC AGC 1379

 E S V R R T R S K I G F G I L L S K E P 136
 GAG TCG GTG CGG CGA ACG CGC AGC AAG ATC GGC TTC GGC ATC CTG CTC AGC AAG GAG CCC 1439

 D G V W A Y N R G E H P I F V N S P T L 156
 GAC GGC GTG TGG GCC TAC AAC CGC GGC GAG CAC CCC ATC TTC GTC AAC TCC CCG ACG CTG 1499

 D A P G G R A L V V R K V P P G Y S I K 176
 GAC GCG CCC GGC CGC GGC CTC GTG GTG CGC AAG GTG CCC CCC GGC TAC TCC ATC AAG 1559

FIG.6B

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V F D F E R S G L Q H A P E P D A A D G 196
 GTG TTC GAC TTC GAG CGC TCG GGC CTG CAG CAC GCG CCC GAG CCC GAC GCC GAC GGC 1619

 P Y D P N S V R I S F A K G W G P C Y S 216
 CCC TAC GAC CCC AAC AGC GTC CGC ATC AGC TTC GCC AAG GGC TGG GGG CCC TGC TAC TCC 1679

 R Q F I T S C P C W L E I L L N N P R • 236
 CGG CAG TTC ATC ACC TCC TGC CCC TGC TGG CTG GAG ATC CTC AAC AAC CCC AGA TAG 1739

 TGGCGGCCCGGGAGGGGGTGGGAGGCGGGCCACCGCCACCTGCCGGCCTCGAGAGGGGCCGATGCCCCAGA 1818

 GACACAGCCCCACGGACAAAACCCCCAGATATCATCTACCTAGATTTAATATAAAGTTTTATATATATGGAAT 1897

 ATATATTACTTGTAAATTATGGAGTCATTTTACAATGTAATTATTATGTATGGTGCAATGTGTGTATATGGACAAA 1976

 ACAAGAAAGACGCACCTTTGGCTTATAATTCTTTCAATACAGATATAATTTCTTCTCTCCTCCTCCTTCTTACT 2055

 TTTTATATATATATAAAGAAAATGATACAGCAGAGCTAGGTGGAAAAGCCTGGGTTTGGTGATGGTTTTTGAGATA 2134

 TTAATGCCCAGACAAAAAGCTAATACCAGTCACTCGATAATAAAGTATTCGCATTATAGTTTTTTAAACTGTCTTCT 2213

 TTTTACAAAGAGGGGCAGGTAGGGCTTCAGCGGATTTCTGACCCCATCSTGTACCTTGAAACTTGACCTCAGTTTTCAAG 2292

 TTTTACTTTTATTGGATAAAGACAGACAACAAATTGAAAAGGGAGGAAAGTCACATTTACTCTTAAGTAACCCAGAGAAAG 2371

FIG.6C

TTCTGTTGTTCCCTTCCTGCCCATGGCTATGGGGTGTCCAGTGGATAGGGATGGCGGTGGGGGAAAAGGAGAATACACTGG 2450

CCATTTATCCTGGACAAGCTCTTCCAGTCTGATGGAGGAGGTTCATGCCCTAGCCTAGAAAGGCCCAGGTCCATGACCC 2529

CCATCTTTGAGTTATGAGCAAGCTAAAAGAAGACACTATTTCTCACCATTCTTGGAATGGCCTGGGGAAACAAAGACT 2608

GAAATGGGCCTTGAGGCCACCCTGCTACCTTGCAGAGAACCATCTCGAGCCCCGTAGATCTTTTAGGACCTCCACAGGC 2687

TATTTCCACCCCCAGCCAAAAATAGCTCAGAACTCTGCCCATCCAGGGCTGTATTAATGATTTATGTAAGGCAGATG 2766

GTTTATTTCTACTTTGTAAAAGGGGAAAAGTTGAGGTTCTGGAAGGATAAATGATTTGCTCATGAGACAAAAATCAAGGTT 2845

AGAAGTTACATGGAAATTGTAGGACCAGAGCCATATCATTAGATCAGCTTCTGAAGAAATATTCMAAAAAAGAAAGTC 2924

TCCTTGGCCAGATAACTAAGAGGAATGTTTCATTGTATATCTTTTCTTGGAGATTATATAACATATTAAGTGCTC 3003

TGAGAAAGTCCTGTGTATTATCTCTTGCTGCATAATAAATTATCCCCAACTTAAAAAAAAAAAAAAAAAAAAAACTCGA 3082

G 3083

FIG. 6D

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/02291

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07H 21/02; C12N 15/63, 5/16; A61K 31/70; C07K 14/435

US CL : 514/44; 536/23.1, 23.5; 435/320.1, 325; 530/350

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 536/23.1, 23.5; 435/320.1, 325; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, GENBANK, MEDLINE, SCISEARCH, BIOSIS, EMBASE, CAPLUS, WPIDS

Search terms: fchd7, pfchd7, Falb, D, cardio?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	OHNO et al. Gene Therapy for Vascular Smooth Muscle Cell Proliferation After Arterial Injury. Science. 05 August 1994, Vol. 265, pages 781-784. See entire reference.	33-34
A	PATRICK JR., et al. Shear Stress and Cyclic Strain Modulation of Gene Expression in Vascular Endothelial Cells. Blood Purification. 1995. Vol. 13, pages 112-124. See entire document.	1-8, 33-34
A	SHYY et al. Multiple cis-elements mediate shear stress-induced gene expression. Journal of Biomechanics. December 1995. Vol. 28, No. 12, pages 1451-1457. See entire document.	1-8, 33-34

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 20 MAY 1997	Date of mailing of the international search report 11 JUL 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DEBORAH J. R. CLARK Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/02291

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WEATHERALL. Scope and limitations of gene therapy. British Medical Bulletin. 1995. Vol. 51, No. 1, pages 1-11. See entire document.	33-34

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/02291

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-8 and 33-34

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/02291

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-8 and 33-34, drawn to an isolated polynucleotide, a gene product, and methods of using said polynucleotide or gene product.

Group II, claim(s) 9, 30, and 55, drawn to an antibody and methods of using said antibody.

Group III, claim(s) 10-11, drawn to a transgenic animal.

Group IV, claim(s) 12-20, 35-54, and 69, drawn to diagnostic methods, comprising nucleic acid assays.

Group V, claim(s) 21-29, 31-32, and 63-68, drawn to methods for treatment, comprising administering some compound that modulates an activity.

Group VI, claims 56-62, drawn to methods using binding assays, not specifically utilizing the antibody of group II.

The inventions listed as Groups I-VI do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the first product, an isolated polynucleotide and methods of using said product are so linked as group I. Groups II and III are inventions of separate products, an antibody and a transgenic animal. Groups IV-VI are methods which are not restricted to the use of the product of invention I and represent the multiple uses of the product.